Design, Synthesis, and In Vitro Testing of α-Methylacyl-CoA Racemase Inhibitors

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The enzyme α-methylacyl-CoA racemase (AMACR) is overexpressed in prostate, colon, and other cancers and has been partially validated as a potential therapeutic target by siRNA knockdown of the AMACR gene. Analogs of the natural substrate branched chain α-methylacyl coenzyme A esters, possessing one or more *β*-fluorine atoms, have been synthesized using Wittig, conjugate addition, and asymmetric aldol reactions and found to be reversible competitive inhibitors. Each diastereomer of the previously reported inhibitor ibuprofenoyl-CoA was also tested. The compounds had K_i values of 0.9−20 μM and are the most potent inhibitors yet known. The presence of *β*-fluorine on the α-methyl group or the acyl chain results in a significant lowering of the K_i value compared with nonfluorinated analogs, and this is attributed to a lowering of the pK_a of the α-proton, facilitating enolization and binding. Several of the CoA ester inhibitors were formed by incubating the free carboxylic acid precursors with cell free extracts and CoA. α-Trifluoromethyltetradecanoic acid, the precursor to the most potent inhibitor, was shown to inhibit growth of cancer cell lines PC3, CWR22 Rv1, and Du145 in a dose-dependent manner and could be related to the expression level of AMACR.

Introduction

Prostate cancer (PCa[a]) is the most common solid tumor malignancy in men in Western countries and was responsible for 30 000 deaths during 2005 in the U.S.[1] Androgen deprivation by surgery or use of drugs is the most common treatment for advanced PCa. However, despite a rapid initial response in the majority of cases, PCa will ultimately progress to a hormone refractory stage and metastatic disease. There is, therefore, a great deal of interest in the identification of improved diagnostic markers and therapeutic agents. Through mRNA expression profiling, it has been shown that the α-methylacyl-CoA racemase gene (AMACR) is consistently upregulated in prostate cancer (8-fold increase in mRNA), and overexpression of the enzyme has been confirmed.[2,3] AMACR is involved in the *β*-oxidation of branched-chain fatty acids, and recent data show that the entire peroxisomal pathway for branched-chain substrates is up-regulated.[4] AMACR overexpression starts early in oncogenesis, with 70% of high-grade prostatic intraepithelial neoplasia staining positive. In primary PCa, >95% of cases are positive for AMACR, whereas <1% of adjacent normal epithelial tissue stains positive. Once overexpressed in PCa, the AMACR level remains elevated during progression to higher grades and stages and even metastases. The enzyme is also overexpressed in other cancers such as colorectal (92% of cases) and breast (44% of cases).[5] Epidemiological studies have shown that red meat and dairy products, major sources of branched-chain fatty acids, are associated with increased prostate cancer risk.[6] Genome-wide scans for linkage in hereditary PCa families implicate 5p13, the chromosomal region of AMACR, as the location of the prostate cancer susceptibility gene, and it has recently been reported that sequence variants of AMACR may be responsible for increased prostate cancer risk.[7] Two specific mutations, S52P and L107P, in peroxisomal AMACR are thought to be responsible for adult-onset sensory motor neuropathy. Patients with the disease show elevated plasma concentrations of pristanic acid and C27-bile acid intermediates.[8] Recent data has shown that AMACR is functionally important in the growth of PCa cells. Small interference RNA (siRNA) knockdown of the AMACR gene reduced the expression of AMACR and significantly impaired the proliferation of the androgen responsive PCa cell line LAPC-4. Simultaneous inhibition of both the AMACR pathway by siRNA and androgen signaling by androgen withdrawal or antiandrogen suppressed the growth of LAPC-4 cells to a greater extent than either treatment alone. These results confirm that AMACR is an androgen-independent growth modifier in prostate cancer and suggest that the enzyme is a complementary drug target with androgen ablation therapy.[9] There are currently no effective small molecule drug treatments for hormone refractory prostate cancer. The prospects for drug treatment with AMACR inhibitors are good because, although AMACR exists in many organs, the major clinical manifestation of congenital racemase deficiency is adult-onset sensory motor neuropathy, which is caused by the slow, prolonged accumulation of branched-chain fatty acids.[8,10] During treatment with AMACR inhibitors, this could be prevented by a low phytanic acid diet. AMACR is also involved in bile acid synthesis, however, an alternative pathway that does not rely on AMACR has recently been demonstrated in AMACR-deficient mice.[11]

AMACR is a peroxisomal and mitochondrial enzyme catalyzing the racemisation of branched chain α-methylacyl-CoA esters such as pristanoyl-CoA **1** and the bile acid intermediate 3α,7α,12α-trihydroxycholestanoyl-CoA (THC-CoA) **2**

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[a] Abbreviations: AMACR, α-methylacyl-CoA racemase; CoA, coenzyme A; PCa, prostate cancer; THC, trihydroxycoprostanoyl; DAST, diethylaminosulfur trifluoride; CDI, carbonyl diimidazole; MEM, methoxyethoxymethyl; MOPS, 3-(*N*-morpholino)propanesulfonic acid; LACS, long-chain acyl-CoA synthetases; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC3, CWR22Rv1, Du145, human prostate cancer cell lines; PrEC, prostate epithelial cells; RIPA, radioimmunoprecipitation assay.

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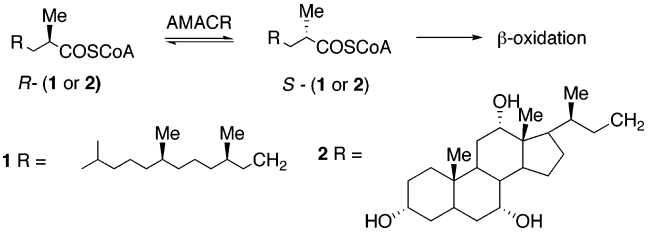


Figure 1. Equilibration of the α-chiral center of pristanoyl-CoA **1** and trihydroxycholestanoyl-CoA **2** by AMACR.

(Figure 1). Pristanoyl-CoA **1** is derived from the diet either directly or by α-oxidation of dietary phytanic acid and exists as (2*R*)- and (2*S*)-stereoisomers. THC-CoA is formed from cholesterol as the (25*R*)-stereoisomer. The racemase interconverts *R*- and *S*-stereoisomers of these α-methylacyl-CoA esters, providing the (*S*)-isomer for the branched-chain acyl-CoA oxidase in the first step of *β*-oxidation.

Little is known about the mechanism of action of this enzyme, which has been purified and characterized by Schmitz et al.[12,13] Based on the observation that the enzyme catalyzes the rapid exchange of the substrate α-proton (2-^{3}H-pristanoyl-CoA) with water, it has been assumed that the enzyme functions by proton abstraction to form an enolate, followed by reprotonation using a one- or two-base mechanism. The nature of the basic groups involved in the proton abstraction has not yet been established, although a crystal structure of *Mycobacterium tuberculosis* AMACR (43% sequence identity with the human enzyme) has recently been determined.[14] Four key residues, Arg91, His126, Asp156, and Glu241, were found to be essential for catalysis, with Asp156 being identified as the putative base.

Prior to the publication of the bacterial crystal structure, we designed a series of substrate analogs **3**−**7** as mechanism-based inhibitors containing one or more fluorine atoms in a *β*-position to the CoA thioester (Figure 2). We envisaged that elimination of fluoride from the proposed enolate by an E1cB mechanism within the active site would generate an α,*β*-unsaturated thioester that may then undergo attack by an active site base or nucleophile to give the inactivated enzyme.

Results and Discussion

Chemistry. The synthesis of compound **3** commenced with a Wittig reaction of dodecyl phosphonium bromide ylid with methyl trifluoropyruvate (Scheme 1) to give a 1:1; *E*/*Z* mixture of the 2-trifluoromethylester **9**. Hydrogenation and ester hydrolysis followed by activation of the carboxylic acid with ethyl chloroformate to form the mixed anhydride allowed coupling with coenzyme A to give the thioester **3**, which was purified by reverse phase HPLC. The synthesis of difluoromethyl thioester **4** was initiated by reacting dodecyl magnesium bromide with trifluoromethyl acrylic acid according to Kitazume et al.[15] The resulting difluoroalkene **10** proved unreactive to the reported hydrogenation conditions using sonication and required successive hydrogenations using Pd/C catalyst at 20 atm pressure. Upon formation of the saturated difluoroacid, carbonyl diimidazole (CDI)-mediated coupling with CoA generated the target thioester **4** in 20% yield after purification by HPLC.

Attempted lipase-mediated resolution of the carboxylic acid precursors to **3** and **4** did not give useful enantioselectivity, so the CoA esters were made from the racemic acids and tested as diastereoisomeric mixtures. For the synthesis of compounds **5** and **6**, we employed an Evans asymmetric aldol reaction to have control over absolute and relative stereochemistry of the 2-methyl and 3-fluoro substituents (Scheme 2). Thus, asymmetric aldol reaction between the boron enolate derived from oxazolidinone **11** and the tetradecanal afforded the *syn* aldol product **12** as a single isomer. S_N2 displacement of the hydroxyl group with fluorine to give **13** was achieved with diethylaminosulfur trifluoride (DAST) at −78 °C. The fluorination proved to be particularly low yielding (20%), a consequence of the propensity for elimination of fluoride, which could be limited at low temperature. Attempts to use TBAF displacement of the mesylate derived from alcohol **12** resulted in almost complete elimination. Removal of the oxazolidinone was achieved in good yield without elimination of fluorine using lithium hydroperoxide to give **14**. CDI-mediated coupling with CoA then afforded the α-methyl-*β*-fluoro compound **5**. The diastereomeric inhibitor **6** was made similarly starting from the alternative oxazolidinone auxiliary **15**.

The THC analogue **7** was made using a similar approach (Scheme 3). In this case, it was necessary to protect the hydroxyl groups in the starting material, cholic acid **16**, such that they could be removed under conditions that would leave the penultimate α-methyl-*β*-fluoro acid **19** intact. After a number of attempts with protecting groups such as tetrahydropyranyl and benzyl, where we were unable to achieve complete protection, we found the methoxyethoxymethyl (MEM) group to be the ideal choice. Thus after the four step conversion of cholic acid to the aldehedye **17**, Evans aldol and DAST fluorination gave compound **18**.

After removal of the auxiliary, the final deprotection to remove the MEM groups was achieved using several drops of concentrated hydrochloric acid (HCl) in tetrahydrofuran (THF) to give the acid **19**. Alternative conditions such as $TiCl_4$ resulted in total decomposition, $ZnBr_2$ gave no reaction, and generation of HCl by use of an acetyl chloride/MeOH mixture resulted in re-esterification of the carboxylic acid. We were unable to achieve the CoA ester coupling using the CDI method, therefore, we used an enzymatic approach that involved incubation of the acid **19** with purified rat liver microsomes in the presence of ATP, $MgCl_2$, and CoA.

Enzyme Inhibition Studies. Enzyme inhibition assays were performed based on the ability of the compounds to inhibit the AMACR-catalyzed racemisation of (25*S*)-THC-CoA **2**. As the AMACR source, an organellar pellet prepared by centrifugation of homogenized rat liver was used. The incubations consisted of 0.005 mg/mL of protein, 100 mM MOPS, pH 7.0, and 50 *μ*M (25*S*)- or (25*R*)-THC-CoA, with increasing concentrations of the inhibitor compounds (0−200 *μ*M dissolved in 20 mM MES, pH 6.0). Reactions were allowed to proceed for 20 min at 37 °C and then terminated by the addition of HCl, followed by resolution of (25*S*)- or (25*R*)-THC-CoA by reversed-phase HPLC.[16] Although our compounds had been designed as mechanism-based irreversible inhibitors, our kinetic data showed that the compounds (**3**−**7**) were actually competitive inhibitors, with inhibition constants in the range 0.9−20 *μ*M (Table 1).

Figure 3 shows a kinetic plot for inhibitor **6** clearly showing the classical profile for reversible competitive inhibition. These are encouraging values, because the only previous reported K_i for an inhibitor of AMACR is 56 *μ*M for ibuprofenoyl-CoA **20**.[12] We assume that this was measured using a diastereomeric mixture made from racemic ibuprofen. We also assayed each diastereomer of ibuprofenoyl-CoA separately and found (*R*)-ibuprofenoyl-CoA **20** to be around 3.5-fold more effective as inhibitor (K_i = 5.4) than the (*S*)-isomer **20** (K_i = 19.2).[17]

Our results show that incorporation of fluorine in the *β*-position to the CoA ester gives compounds that can inhibit AMACR, with the best inhibitor **3** containing the α-trifluoromethyl group. Compound **4** containing the α-difluoromethyl group

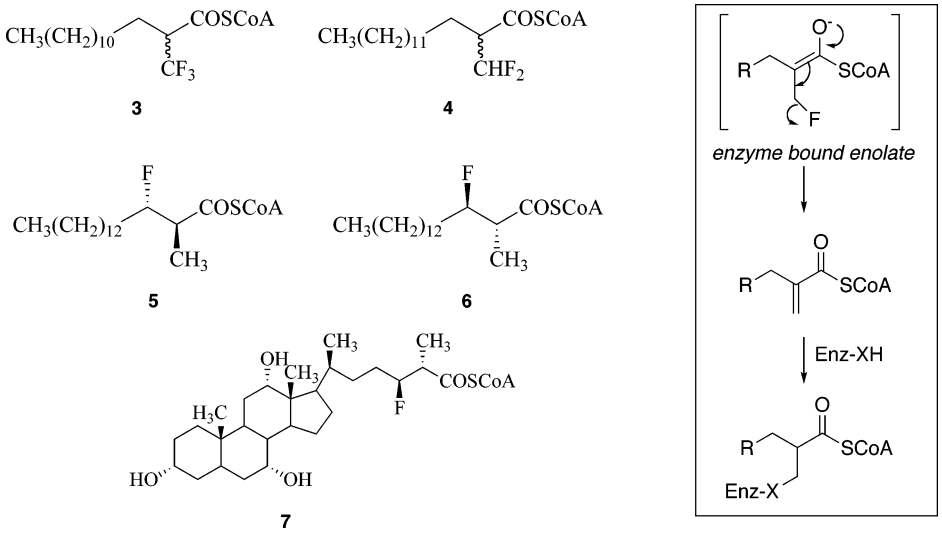
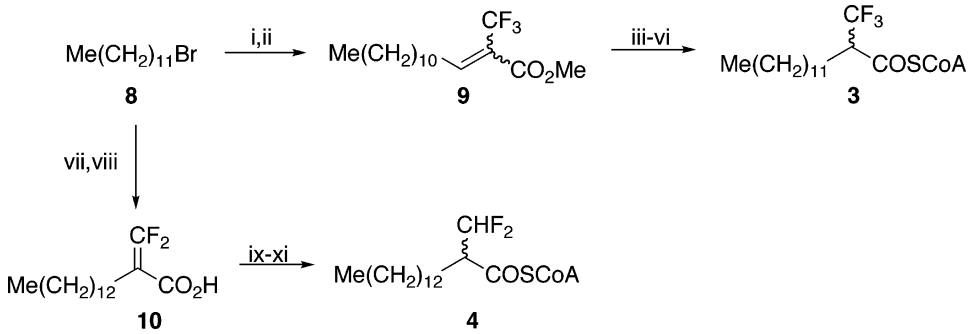


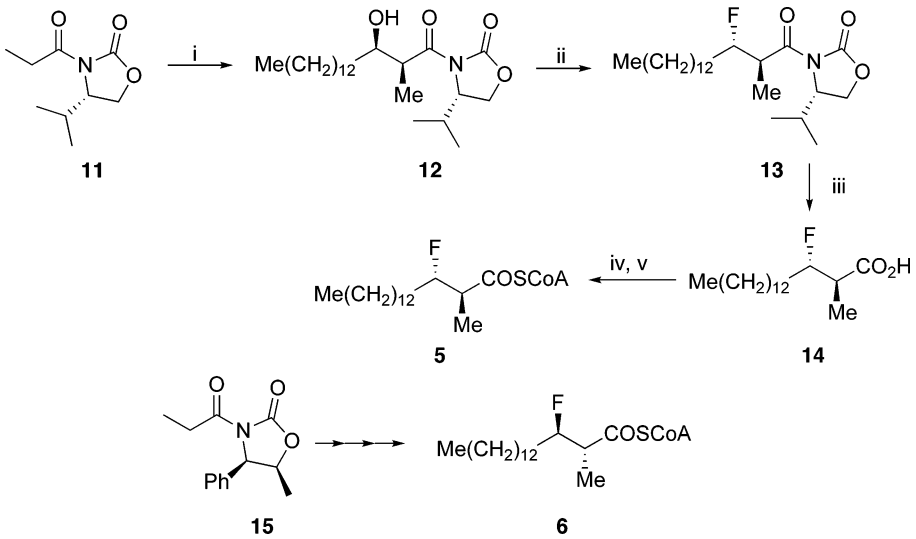
Figure 2. Compounds designed as mechanism-based inhibitors of AMACR, with the proposed mechanism shown in the inset.

Scheme 1[a]



[a] Reagents and conditions: (i) PPh_3, toluene, Δ, 48 h, 98%; (ii) *n*-BuLi, $F_3C(O)CO_2Me$, THF, −78 °C, 3 h, 54%; (iii) H_2/Pd/C, MeOH, rt, 24 h, 94%; (iv) 5 N NaOH, THF, MeOH, rt, 24 h, 97%; (v) NEt_3, $ClCO_2Et$, DCM, rt, 2 h; (vi) $CoALi_3$, $KHCO_3$, *t*-BuOH, rt, 24 h; (vii) Mg turnings, THF, rt, 30 min; (viii) trifluoromethyl acrylic acid, THF, 5 h, −40 °C, 35%; (ix) H_2/Pd/C, MeOH, rt, 20 atm., 67%; (x) CDI, THF; (xi) $CoALi_3$, THF, H_2O, rt, overnight, 20%.

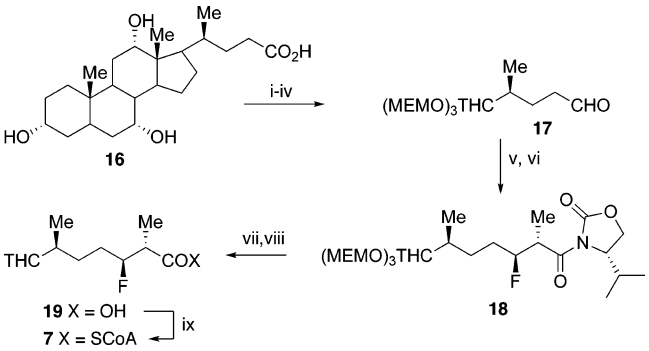
Scheme 2[a]



[a] Reagents and conditions: (i) *n*-Bu_2BOTf, DIPEA, $Me(CH_2)_{12}CHO$, THF, −78 °C, 3 h, 41%; (ii) DAST, DCM, −78 °C, 3 h, 22%; (iii) H_2O_2, $LiOH{\cdot}H_2O$, THF, H_2O, 0 °C, overnight, 90%; (iv) CDI, THF; (v) $CoALi_3$, THF, H_2O, rt, overnight, 20%.

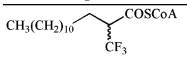
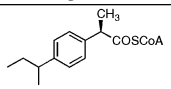
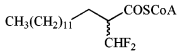
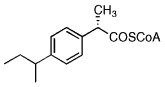
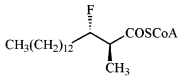
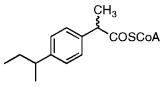
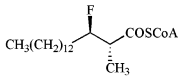
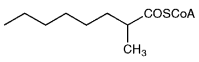
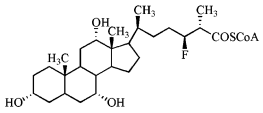
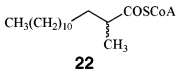
was much less effective for reasons that are as yet unclear. Compounds **5**−**7** containing one fluorine atom on the β-position of the chain have similar low K_i values. The reversal of absolute configuration of the methyl and fluorine groups in compounds **5** and **6** appeared to make little difference to the K_i. The similar K_i of **5** and **6** and the fact that both diastereomers of pristanoyl-CoA **1** are substrates for the racemase led us to believe that stereochemistry at the α-position in this series may not be an important factor. However, the marked difference in K_i values for (*R*)- and (*S*)-ibuprofenoyl-CoA **20** is interesting and probably

Scheme 3[a]



[a] Reagents and conditions: (i) amberlite ion ex. res., MeOH, rt, overnight, 97%; (ii) MEM-Cl, DIPEA, DCM, rt, 24 h, 41%; (iii) $LiAlH_4$, THF, rt, 4 h, 90%; (iv) PCC, DCM, rt, 3 h, 61%; (v) *n*-Bu_2BOTf, DIPEA, CA*i*-Pr, THF, −78 °C, 3 h, 23%; (vi) DAST, DCM, −78 °C, 3 h, 47%; (vii) H_2O_2, LiOH·H_2O, THF, H_2O, 0 °C, overnight, 95%; (viii) conc aq HCl, THF, 3 h, rt, 98%; (ix) rat liver microsomes, ATP, $MgCl_2$, CoASH.

Table 1. Competitive Inhibitors of AMACR[a]

compound	K_i (M)	compound	K_i (M)
 **3**	0.9	 **(*R*)-20**	5.4
 **4**	20	 **(*S*)-20**	19.2
 **5**	1.3	 **20**	56[b]
 **6**	2.3	 **21**	45[b]
 **7**	3.8	 **22**	137[b]

[a] K_i values were determined using direct linear plot analysis. AMACR activity was determined by monitoring the interconversion of (25*R*/*S*)-THC-CoA.[16] Assays consisted of 5 μg/mL protein, 100 mM MOPS pH 7, 50 μM (25*S*) or (25*R*)-THC-CoA with increasing concentrations of inhibitor. [b] K_i for racemic ibuprofenoyl-CoA and K_i values for compounds **21** and **22** calculated from reported kinetic data.[12]

reflects the greater steric demands of the aromatic ring immediately adjacent to the α-carbon.

Schmitz et al. reported an increase in K_m for racemisation of THC-CoA in the presence of 2-methyloctanoyl CoA **21** (270 μM) and 2-methylmyristoyl CoA **22** (175 μM).[12] With the substrate and inhibitor concentrations reported, K_i values of 45 μM for 2-methyloctanoyl **21** and 137 μM for 2-methylmyristoyl CoA **22** can be calculated. The presence of fluorine in our inhibitors clearly has a significant effect, because the analogous fluorinated compounds **3**, **5**, and **6** have K_i values between 0.9 and 2.3 μM. The effect exerted by fluorine is not yet clear, but we hypothesize that the fluorine-induced reduction in pK_a of the α-proton may make these compounds better substrates than

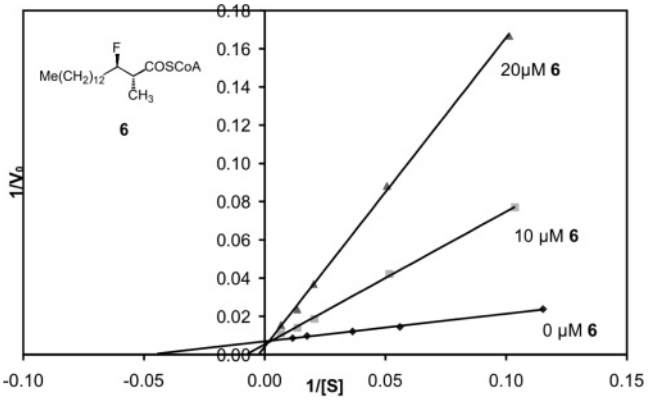


Figure 3. Kinetic plot for inhibitor **6** showing competitive inhibition.

the natural substrate by facilitating enolate formation in the active site, although the K_i for the difluoromethyl compound **4** is anomalous. The polar fluorine-carbon bonds may also increase the binding affinity although, if this were the case, one might expect to see a larger difference in inhibition between inhibitors **3** and **5**−**7**, where C−F bonds would be orientated differently upon binding.

Our inhibitors were conceived prior to the publication of the *M. tuberculosis* crystal structure when we were unaware of the possibility of Asp 152 in human AMACR (Asp 156 in the *M. tuberculosis* structure) acting as the active site base. Because the conjugate addition of an aspartate carboxylate to an unsaturated ester is likely to be reversible, one would not expect mechanism-based irreversible inhibition to be observed. In theory it is possible that our inhibitors eliminate fluorine and that the unsaturated esters are the real inhibitors, but this is unlikely because 2-methylmyrist-2-enoyl CoA is not an inhibitor.[12]

The use of CoA in drug compounds is likely to prevent them from being orally bioavailable. However, the CoA thioesters may be biosynthesized in vivo by a long-chain acyl-CoA thioester synthetases (LACS)[18] or other CoA ester ligases,[19] from the carboxylic acid as a pro-drug. For example, (*R*)-ibuprofen is thioesterified by a stereoselective synthetase in vivo prior to racemisation.[19] Incubation of the free carboxylic acids corresponding to inhibitors **3**, **5**, and **7**, with rat liver homogenate or purified microsomes in the presence of ATP, $MgCl_2$, and CoA indeed showed that all three acids were thioesterified by an endogenous synthetase/ligase. It can be speculated that an acid, which is very similar to the natural substrate precursor, could become thioesterified by a synthetase and would be transported into the peroxisome and/or mitochondrion where AMACR is localized. This in vivo arming of the inhibitor might afford selective toxicity, because the free acid or ester acid pro-drug would not be active as an inhibitor.

Inhibitory Activity of (±)-2-Trifluoromethyltetradecanoic Acid on Prostate Tumor Cell Growth. Several prostate cancer cell lines (PC3, CWR22Rv1, Du145) as well as cultured prostate epithelial cells (PrEC) were incubated with 50 μM and 100 μM (±)-2-trifluoromethyltetradecanoic acid, the carboxylic acid precursor of compound **3**. The cells were then tested for viability at 24 h intervals for up to 10 days using the MTT cell proliferation assay. The results presented in Figure 4a clearly show that this compound has a cytotoxic effect. With both concentrations of the drug tested, the number of viable cells was significantly diminished after 4 days for all cell lines. CWR22Rv1 was the least sensitive to the cytotoxic effect of 2-trifluoromethyltetradecanoic acid, with a viability of 95.3% and 74% after 3 days of incubation with 50 μM and 100 μM of the drug, respectively. Interestingly, this cell line had the lowest

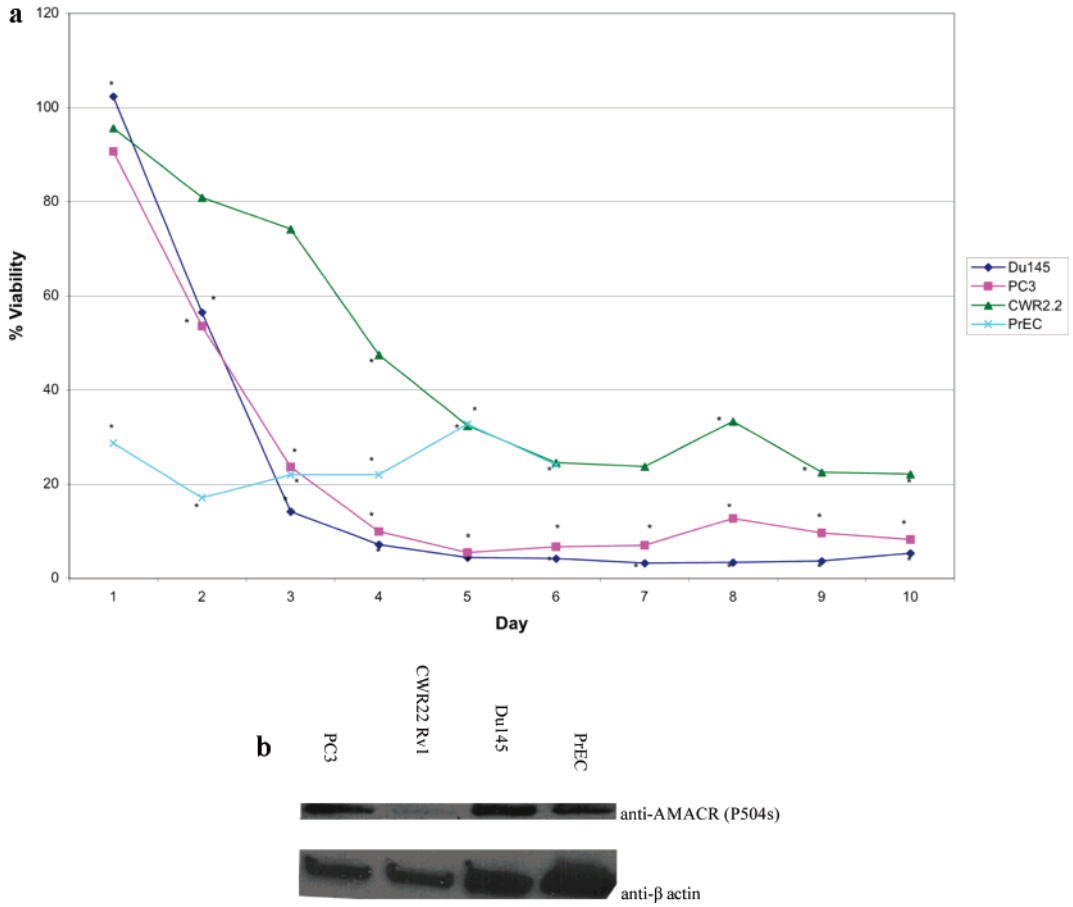


Figure 4. (a) Cell viability for PC3, CWR22 Rv1, Du 145, and PrEC following treatment with 100 μM (±)-2-trifluoromethyltetradecanoic acid. A two-tailed student's T-test was performed for each triplicate day compared to its vehicle control; any time point for which the *p*-value is less that 0.05 is marked with an asterisk (*). (b) Western Blot analysis of AMACR expression. Immunoblot analysis of AMACR in whole cell lysates of PC3, CWR22 Rv1, Du145, and PrEC. An immunoblot for β-actin expression was performed as a loading control.

AMACR expression as seen by Western Blot (Figure 4b), which suggests that the cytotoxic effect of the compound could be AMACR-dependent.

Surprisingly, nonmalignant PrEC cells were most sensitive to the toxic effect of this compound. Previous studies consistently demonstrate very low expression of AMACR in normal prostate epithelium.[20] However, little data exists on the expression of this protein in cultured prostate epithelial cells.[21,22] Our results show that PrEC expresses AMACR at a readily detectable level. Whether the sensitivity we observed for these cells reflects a combination of effects, both compound-mediated as well as the normal senescence program that these cells inevitably progress through, is not clear at present. These data suggest that follow-up studies in nonprostate and nontumorgenic cell lines both with high and with low AMACR expression are necessary to assess the role of this activity in the cytotoxic effects observed.

Conclusion

A range of AMACR substrate analogs containing an α-difluoromethyl, an α-trifluoromethyl, or a fluorine in the β-position on the acyl chain have been synthesized. Synthetic routes were developed that avoided elimination of fluorine at key stages to allow sufficient quantities to be synthesized for the biological assays. Three α-methyl-β-fluoroacyl inhibitors were made as single isomers to test the effect of chirality. These compounds, originally designed as irreversible mechanism-based inhibitors, are promising reversible competitive inhibitors of AMACR and are significantly more potent than the previously reported 2-methyloctanoyl-CoA, 2-methylmyristoyl-CoA, or ibuprofenoyl- CoA. The presence of fluorine is postulated to lower the pK_a of the α-proton facilitating enolization and binding in the active site. (*R*)- and (*S*)-ibuprofenoyl-CoA were tested separately and, unlike the fluorinated inhibitors or the natural substrates, which have no β-branching, showed significant differential activity with the isomer derived from (*R*)-ibuprofen, exhibiting a 3.5-fold lower K_i. (±)-2-Trifluoromethyltetradecanoic acid, the precursor of the most potent AMACR inhibitor **3**, showed cytotoxic activity on cancer cell lines PC3, CWR22 Rv1, and Du145, with CWR22 Rv1, expressing low levels of AMACR, being least affected. Normal prostate epithelial PrEC cells expressing an unexpectedly high level of AMACR were also affected. Although the 100 μM concentration is relatively high, the carboxylic acid needs to be taken up by the cells and activated to its CoA ester prior to targeting the enzyme. The concentration of inhibitor required for substantial cell growth inhibition and time taken to exert its affect are, therefore, not surprising, but these data provide proof of concept for this first series of AMACR inhibitors and encourage us to examine related compounds as potential drug leads in the future.

Experimental Section

General. All solvents were distilled and kept over 3 Å molecular sieves prior to use. Analytical grade solvents were used for reactions involving aqueous media. Dichloromethane (DCM) was distilled from calcium hydride before use. Sureseal anhydrous *N*,*N*-dimethylformamide (DMF) was purchased from Aldrich or distilled from calcium hydride before use. Methanol (MeOH) was distilled from

a magnesium and iodine still for 2−3 h under argon atmosphere before use. THF was dried from sodium and benzophenone until purple coloration persisted and then distilled under atmospheric pressure. Chiral auxiliaries-(*S*)-4-isopropyl-3-propionyloxazolidin-2-one and (4*R*,5*S*)-4-methyl-5-phenyl-3-propionyloxazolidin-2-one were purchased from Fluka. Coenzyme A trithium salt (CoA-Li_3) was purchased from Roche. (*R*)- and (*S*)-Ibuprofen were a kind gift from Knoll Pharmaceuticals, Amsterdam (a branch of BASF Pharma). The CoA esters were synthesized according to Rasmussen et al.[23] All other reagents were purchased from Sigma-Aldrich unless otherwise stated. Analytical thin layer chromatography was performed on 2 × 5 cm aluminum sheets, precoated with Merck Kieselgel 60, and detection by UV 254 nm light. The developed chromatograms were visualized with a UV lamp. Plates were then treated with potassium permanganate dip and developed with a heat gun. Flash column chromatography was performed using silica (Merck 9385 Kieselgel 60). Crude products were applied to the column either dissolved in a small amount of the eluent or absorbed onto silica, and then the column was eluted with additional solvent of increasing polarity. Fractions of 5−100 mL were collected and analyzed by TLC. ^{1}H, ^{13}C, COSY, and HMQC NMR spectra were recorded on a Bruker AMX-400 machine. Coupling constants (*J* values) are reported in Hz. Chemical shifts are given in ppm, downfield from an internal standard of tetramethylsilane (TMS). IR spectra were recorded as nujol mulls in the range 4000−600 cm^{-1} using a Perkin-Elmer 883 spectrophotometer and a Perkin-Elmer FT-IR Paragon 1000 spectrometer on sodium chloride plates washed with ethanol. Mass spectrometry analyses (ES^+, ES^-, EI^+) were performed on a Trio1000 spectrometer and run by the mass spectrometry service within the University of Liverpool. Where chemical ionizations (CI) were used, ammonia was used as the carrier gas. Gas chromatography was carried out on a Shimadzu GC-14B module using an EC-1 column (30 m × 0.32 mm, 1 μm film thickness). Chiral separations used a FS-Lipodex-E column (25 m × 0.25 mm). Analytical reverse phase HPLC was performed on a Waters 2695 separations module equipped with a Waters 996 photodiode array detector. Separations were carried out using an Alltech Altima C-18 5μ, 250 mm × 4.6 mm column packed with 5 μm spherisorb ODS silica, with a gradient using 90% MeOH/H_2O and 50% MeOH/25 mM K_2HPO_4/KH_2PO_4. Analyses were also carried out using a Perkin-Elmer Biocompatible Binary Pump 250 and a Pharmacia UV detector LKB-UV-MII. Separations were carried out using a SupelcoSIL LC-18-DB column 250 mm × 4.6 mm, the gradient used was 40 to 70% A/B; (A = 70% MeCN/16.9 mM sodium phosphate, pH 6.9; B = 10% MeCN/16.9 mM sodium phosphate, pH 6.9).

Methyl 2-Trifluoromethyltetradec-2-enoate (9). Triphenylphosphine (5.41 g, 20.66 mmol, 1 equiv) was added to a solution of 1-bromododecane (5 mL, 20.66 mmol, 1 equiv) in toluene (50 mL). The solution was heated under reflux for 48 h. The solvent was then removed under reduced pressure to yield dodecyltriphenylphosphonium bromide (8.74 g, 98%) as a dark yellow oil, which was used without further purification. *n*-Butyllithium (1.6 M, 3.72 mL, 5.95 mmol, 1.1 equiv) was added dropwise to a cooled solution of dodecyltriphenylphosphonium bromide (2.33 g, 5.41 mmol, 1 equiv) in THF (50 mL) at −70 °C. The mixture was allowed to warm to room temperature over 2 h and stirred for an additional 1 h. The dark red mixture was again cooled to about −70 °C, and methyl trifluoropyruvate (843 mg, 5.41 mmol, 1 equiv) was added dropwise. The resulting yellow solution was monitored by TLC until conversion was complete. The mixture was then warmed to room temperature and filtered through a short column of silica gel (30 g) to remove triphenylphosphine oxide and salts. The crude product was concentrated in vacuo and chromatographed on silica gel (eluent: Et_2O/hexane (1:20) to yield both geometric isomers (900 mg, 54%) of methyl 2-trifluoromethyltetradec-2-enoate **9** in an approximate 1:1 ratio; R_f = 0.68 (*E*-isomer), 0.62 (*Z*-isomer), Et_2O/hexane (1:20).

2-Trifluoromethyltetradecanoyl-CoA (3). To a solution of methyl 2-trifluoromethyltetradec-2-enoate **9** (890 mg, 2.88 mmol) in distilled MeOH (10 mL) was added 1 spatula of palladium over activated carbon. The vessel was then evacuated under reduced pressure and flushed with hydrogen three times. The reaction was allowed to stir overnight. The mixture was then flushed with nitrogen and opened to the atmosphere. The mixture was filtered through a pad of Celite and washed with MeOH. The solvent was then concentrated in vacuo to yield methyl 2-trifluoromethyltetradecanoate as a pale yellow oil (837 mg, 94%). Saponification was carried out by dropwise addition of 5 N sodium hydroxide (1 mL) to a solution of methyl 2-trifluoromethyltetradecanoate (70 mg, 0.23 mmol) in THF (3 mL)/MeOH (2 mL). The resulting yellow solution was allowed to stir at room temperature overnight. After acidification with 1 M HCl (5 mL), the crude product was redissolved in DCM (10 mL) and extracted with water (2 × 5 mL). The organic layer was dried ($MgSO_4$), concentrated in vacuo, and chromatographed on silica gel (eluent: Et_2O/hexane (2:5)) to yield 2-trifluoromethyltetradecanoic acid as a pale yellow oil (61.4 mg, 91%): ^{1}H NMR (400 MHz, $CDCl_3$) 0.88 (3H, t, J = 7.0 Hz, H-14), 1.22−1.36 (20H, m, H-4 to H-13), 1.72−1.78 (1H, m, H-3), 1.83−1.92 (1H, m, H-3), 3.06−3.14 (1H, m, H-2); ^{13}C NMR (100 MHz, $CDCl_3$) 14.4 (C-14), 23.0−32.3 (C-3 to C-13), 50.8 (C-2), 168.5 (C-1); ν_{max} /cm^{-1} 1756 (C═O); ν_{max}/cm^{-1} 3300−2950 (O−H acid), 1729 (C═O); HRMS calcd for $C_{15}H_{31}F_3O_2N$ (M + NH_4^+), 314.2307; found, 314.2315. To a stirred solution of carbonyldiimidazole (1.84 mg, 11.35 μmol, 1.2 equiv, desiccated over silica crystals overnight) in THF (0.2 mL) was added a solution of 2-trifluoromethyltetradecanoic acid (2.81 mg, 9.45 μmol, 1 equiv) in THF (0.2 mL). After 2 h of stirring at room temperature, a solution of coenzyme A trilithium salt (CoA-Li_3; 8.2 mg, 10.39 μmol, 1.1 equiv) in water (0.2 mL) was added. The resulting solution was allowed to stir at room temperature overnight. Two drops of 0.1 M HCl was then added, and the solution was diluted with MeOH (0.5 mL). The product was then purified by semi-preparative reverse phase HPLC (elution gradient MeOH/H_2O/25 mM phosphate buffer) to give the title compound **3** as a white amorphous solid (1.89 μmol, 20% yield, calculated from UV absorption of a specific concentration): UV/vis absorption maxima at 260 nm; HRMS calculated for $C_{36}H_{60}F_3N_7O_{17}P_3S$ (M + H^-), 1044.2982; found, 1044.2931 (for HPLC chromatographs, see Supporting Information, S10).

2-Difluoromethylenepentadecanoic Acid (10). 1-Bromododecane (3.25 mL, 13.45 mmol, 2.5 equiv) and magnesium turnings (325 mg, 13.45 mmol, 1 equiv) were added to a solution of trifluoromethylacrylic acid (750 mg, 5.38 mmol, 1 equiv) and trimethylsilyl chloride (0.68 mL, 5.38 mmol, 1 equiv) in THF (20 mL) at −40 °C. After stirring for 5 h at that temperature, the mixture was quenched with saturated ammonium chloride (20 mL) below −20 °C. The resulting solution was extracted with ether (100 mL). The combined extracts were then washed with water (100 mL) and dried over anhydrous $MgSO_4$. The solvent was removed and the crude product was chromatographed on silica gel (eluent: EtOAc/hexane (1:4)) to yield 2-difluoromethylenepentadecanoic acid **10** as an amorphous yellow solid (550 mg, 35%): R_f = 0.46 EtOAc/hexane (3:7).

2-Difluoromethylpentadecanoyl-CoA (4). A solution of **10** (160 mg, 0.55 mmol) in distilled MeOH (5 mL) containing 1 spatula of palladium over activated carbon was hydrogenated at a pressure of 20 atm for 16 h. HCl (1 M, 2 mL) was added, and the mixture was filtered through a pad of Celite. The crude product was concentrated in vacuo and chromatographed on silica gel (eluent: EtOAc/hexane (1:4)) to yield 2-difluoromethylpentadecanoic acid as a pale yellow oil (108 mg, 67%): R_f = 0.52 EtOAc/hexane (3:7); δ ^{1}H (400 MHz, $CDCl_3$) 0.88 (3H, t, J = 7.0 Hz, H-15), 1.22−1.38 (20H, m, H5−14), 1.42−1.51 (2H, m, H-4), 1.71−1.78 (2H, m, H-3), 2.82−2.89 (1H, m, H-2), 5.96 (1H, td, J_{H-F} = 85.7 Hz, $J_{2,3}$ 5.2 Hz, C*H*F_2); δ ^{13}C (100 MHz, $CDCl_3$) 14.4 (C-15), 23.0−30.1 (C-4 to C-12), 32.2 (C-3), 50.2 (C-2), 116.1 (CF_2, t, J = 240 Hz), 168.3 (C1); HRMS calcd for $C_{16}H_{29}F_2O_2$ (M + H^+), 310.4022; found, 310.4008. 2-Difluoromethylpentadecanoic acid (2.81 mg, 9.58 μmol) was reacted according to the procedure previously described above for 2-trifluoromethyltetradecanoic acid to afford the title compound **4** as a crystalline solid (1.89

μmol, 20%): UV/vis- absorption maxima at 260 nm; HRMS calcd for $C_{38}H_{63}F_3N_7O_{18}P_3S$, 1068.3034 (M + H^- + MeOH); found, 1068.3028 (for HPLC chromatographs see Supporting Information S10).

***N*-(2*S*,3*R*,4′*S*)-3-Hydroxy-2-methylhexadecanoyl)-4′-isopropyloxazolidin-2′-one (12).** *n*-Dibutylboron triflate (1.1 M in DCM, 2.02 mL, 2.05 mmol, 1.4 equiv) and diisopropylethylamine (0.41 mL, 2.35 mmol, 1.6 equiv) were added successively to a solution of (*S*)-4-isopropyl-3-propionyloxazolidin-2-one **11** (0.35 mL, 2.05 mmol, 1.4 equiv) in DCM (5 mL) at −78 °C. After stirring for 30 min, a solution of tetradecanal (313 mg, 1.46 mmol, 1 equiv) in DCM (2 mL) was added dropwise. The resulting pale yellow solution was then allowed to stir at −78 °C for an additional 30 min and then 2 h at room temperature. The solution was quenched with 0.1 M phosphate buffer (pH 7.2, 5 mL). The organic layer was extracted with 1 M HCl (5 mL), $NaHCO_3$ (5 mL), and brine (5 mL). The organic layer was dried ($MgSO_4$), concentrated in vacuo and chromatographed on silica gel (eluent: Et_2O/hexane (1:1)) to yield *N*-(2*S*,3*R*,4′*S*)-(3-hydrox-2-methylhexadecanoyl)-4′-isopropyloxazolidin-2′-one **12** as a clear oil (236 mg, 41%): R_f = 0.15 Et_2O/hexane (1:1).

***N*-(2*R*,3*S*,4′*S*)-(3-fluoro-2-methylhexadecanoyl)-4′-isopropyloxazolidin-2′-one (13).** *N*-(2*S*,3*R*,4′*S*)-3-Hydroxy-2-methylhexadecanoyl)-4′-isopropyloxazolidin-2′-one **12** (30 mg, 75.5 mmol) in DCM (2 mL) was added dropwise to a 0.5 M solution of DAST (0.15 mL, 1 equiv) in DCM (1.2 mL) at −78 °C. After 2 h, the solution was allowed to warm to room temperature and an equal volume of water was carefully added. The organic layer was washed with $NaHCO_3$ (1 mL) and water (1 mL), dried ($MgSO_4$), concentrated in vacuo, and chromatographed on silica gel (eluent: EtOAc/hexane (1:15)) to yield *N*-(2*R*,3*S*,4′*S*)-(3-fluoro-2-methylhexadecanoyl)-4′-isopropyl-oxazolidin-2′-one **13** as a pale yellow oil (6.7 mg, 22%): R_f = 0.67 Et_2O/hexane (1:1).

(2*R*,3*S*)-3-fluoro-2-methylhexadecanoic Acid (14). Hydrogen peroxide (30% v/v, 1.2 μL, 6 equiv) was added dropwise to a stirred solution of **13** (6.7 mg, 16.8 μmol) in THF (0.5 mL) at 0 °C. Lithium hydroxide monohydrate (2 equiv) was then added, and the mixture was allowed to stir at room temperature overnight. Saturated sodium sulfite (0.5 mL) was added, followed by 1 M HCl (0.5 mL). The crude product was then redissolved in DCM (5 mL) and extracted with water (2 × 4 mL). The organic layer was dried ($MgSO_4$), concentrated in vacuo, and chromatographed on silica gel (eluent: EtOAc/hexane (1:3)) to yield (2*R*,3*S*)-3-fluoro-2-methylhexadecanoic acid **14** as a clear oil (4.35 mg, 90%): R_f = 0.33 Et_2O/hexane (1:1).

(2*R*,3*S*)-3-Fluoro-2-methylhexadecanoyl-CoA (5). (2*R*, 3*S*)-3-Fluoro-2-methylhexadecanoic acid **14** (3.1 mg, 7.76 μmol) was reacted according to the procedure described above for 2-trifluoromethyltetradecanoic acid to yield the title compound **5** (1.89 μmol, 26% yield, calculated from UV absorption of a specific concentration): UV/vis absorption maxima at 260 nm; HRMS calcd for $C_{38}H_{63}FN_7O_{17}P_3S$ (M + H^-), 1036.3423; found, 1036.3433 (for HPLC chromatographs, see Supporting Information S10).

(3α,7α,12α)-Tris(methoxyethoxymethyl)-5β-cholestan-24-al (17). Amberlite ion-exchange resin (20 g, prewashed with acetone, 1 M HCl, water) was added to a solution of cholic acid **16** (7.48 g, 18.33 mmol) in MeOH. The resulting mixture was allowed to stir at room temperature overnight. The crude product was filtered, redissolved in hot ethyl acetate (100 mL), refiltered, and concentrated in vacuo to yield to methyl cholate as a white crystalline solid (7.52 g, 97%): R_f = 0.11 DCM/MeOH (9:1). Methoxyethoxymethyl chloride (3.98 mL, 34.9 mmol, 7 equiv) was added dropwise to a solution of methyl cholate (2.1 g, 4.99 mmol, 1 equiv) and diisopropylethylamine (6.1 mL, 34.9 mmol, 7 equiv) in DCM (30 mL). The resulting solution was allowed to stir at room temperature overnight. The reaction was quenched with saturated ammonium chloride (20 mL) and extracted with brine (2 × 30 mL). The organic layer was dried ($MgSO_4$), concentrated in vacuo, and chromatographed on silica gel (elution: DCM/MeOH (9:1)) to yield methyl (3α,7α,12α)-tris(methoxyethoxymethyl) cholate as a pale yellow oil (1.41 g, 41%): R_f = 0.5 DCM/MeOH (9:1). Lithium aluminum hydride (3.5 M, 4 equiv, 6.88 mL, 24.1 mmol) was added dropwise to a solution of methyl (3α,7α,12α)-tris(methoxyethoxymethyl) cholate (1.49 g, 3.53 mmol) in THF (35 mL). The resulting suspension was allowed to stir at room temperature for 4 h. The reaction was quenched by the addition of water (5 mL), followed by 1 M sodium hydroxide solution (2 mL) and water (5 mL). The resulting precipitate was filtered, and the filtrate was concentrated in vacuo. The crude was then chromatographed on silica gel (eluent: EtOAc/hexane (1:8) to yield (3α,7α,12α)-tris(methoxyethoxymethyl)-5β-cholestan-24-ol as a clear oil (606 mg, 45%): R_f = 0.26 DCM/MeOH (9:1). (3α,7α,-12α)-Tris(methoxyethoxymethyl)-5β-cholestan-24-ol (606 mg, 0.92 mmol, 1 equiv) was added to a solution of pyridinium chlorochromate (397 mg, 1.84 mmol, 2 equiv) in DCM (9 mL). The resulting solution was allowed to stir at room temperature for 3 h. The reaction was quenched with ethanol (2 mL) and filtered through a silica pad. The crude was concentrated in vacuo and chromatographed on silica gel (eluent: EtOAc/hexane (1:4)) to yield (3α,7α,12α)-tris(methoxyethoxymethyl)-5β-cholestan-24-al **17** as a clear oil (367 mg, 61%): R_f = 0.36 DCM/MeOH (9:1).

***N*-(24*S*,25*R*,4′*S*)-(3α,7α,12α)-Tris(methoxyethoxymethyl)-24-fluoro-5β-cholestan-26-oyl-4′-isopropyl-oxazolidin-2′-one (18).** (3α,7α,12α)-Tris(methoxyethoxymethyl)-5β-cholestan-24-al **17** (367 mg, 0.56 mmol) was reacted according to the procedure described above (for **12**) to yield *N*-(24*R*,25*S*,4′*S*)-(3α,7α,12α)-tris(methoxyethoxymethyl)-24-hydroxy-5β-cholestan-26-oyl-4′-isopropyl-oxazolidin-2′-one as a pale yellow oil (109 mg, 23%): R_f = 0.59 DCM/MeOH (9:1). *N*-(24*R*,25*S*,4′*S*)-(3α,7α,12α)-tris(methoxyethoxymethyl)-24-hydroxy-5β-cholestan-26-oyl-4′-isopropyloxazolidin-2′-one (109 mg, 0.13 mmol) was reacted according to the procedure described above (for **13**) to yield *N*-(24*S*,25*R*,4′*S*)-(3α,7α,12α)-tris(methoxyethoxymethyl)-24-fluoro-5β-cholestan-26-oyl-4′-isopropyl-oxazolidin-2′-one **18** as a pale yellow oil (51 mg, 47%): R_f = 0.3 DCM/MeOH (9:1).

(24*S*,25*R*)-(3α,7α,12α)-Trihydroxy-24-fluoro-5β-cholestan-26-oic Acid (19). *N*-(24*S*,25*R*,4′*S*)-(3α,7α,12α)-Tris(methoxyethoxymethyl)-24-fluoro-5β-cholestan-26-oyl-4′-isopropyloxazolidin-2′-one **18** (44.5 mg, 61 mmol) was reacted according to the procedure described above (for **14**) to yield (24*S*,25*R*)-(3α,7α,12α)-tris(methoxyethoxymethyl)-24-fluoro-5β-cholestan-26-oic acid as a clear oil (41 mg, 92%): R_f = 0.21 DCM/MeOH (9:1). Concentrated HCl (five drops) was added to a solution of (24S,25R)-(3α,7α,-12α)-tris(methoxyethoxymethyl)-24-fluoro-5β-cholestan-26-oic acid (12 mg, 16.4mmol) in THF (5 mL). The resulting solution was allowed to stir at room temperature for 2 h. The solvent was concentrated in vacuo, and the crude residue was triturated with DCM (2 mL) to yield (24*S*,25*R*)-(3α,7α,12α)-trihydroxy-24-fluoro-5β-cholestan-26-oic acid (**19**) as a clear oil (5 mg, 65%): R_f = 0.07 DCM/MeOH (9:1).

(24*S*, 25*R*)-(3α,7α,12α)-Trihydroxy-24-fluoro-5β-cholestan-26-oyl-CoA (7). The CoA ester (24*S*,25*R*)-(3α,7α,12α)-trihydroxy-24-fluoro-5β-cholestan-26-oyl-CoA, **7**, was synthesized using purified rat liver microsomes. The microsomes were prepared by differential centrifugation of a rat liver homogenate as previously described.[24] One millimolar of compound **19** dissolved in 10 mM Tris pH 8.0 and 1 mg/mL β-cyclodextrin was incubated in 100 mM Tris pH 8.0, 10 mM ATP, 10 mM $MgCl_2$, 1 mM CoA, and 0.5 mM DTT with 0.3 mg/mL microsomal protein for 6 h at 37 °C. The synthesized CoA ester was purified by HPLC[8] (for HPLC chromatographs, see Supporting Information S10).

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Supporting Information Available: Experimental and analytical data for **6** and spectroscopic data for compounds **3**−**5** and **7** and all synthetic intermediates, HPLC traces for CoA esters **3**−**7**, the AMACR inhibition and cytotoxicity assays, and Western Blot analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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