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# New Molecular Markers for Prostate Tumor Imaging: A Study on 2-Methylene Substituted Fatty Acids as New AMACR Inhibitors**

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**Abstract:** The development of prostate carcinoma is associated with alterations in fatty acid metabolism. α-Methylacyl-CoA racemase (AMACR) is a peroxisomal and mitochondrial enzyme that catalyses interconversion between the (*S*)/(*R*)-isomers of a range of α-methylacyl-CoA thioesters. AMACR is involved in the β-oxidation of the dietary branched-chain fatty acids and bile acid intermediates. It is highly expressed in prostate (more than 95%), colon (92%), and breast cancers (44%) but not in the respective normal or hyperplastic tissues. Thus, targeting of AMACR could be a new strategy for molecular imaging and therapy of prostate and some other cancers. Unlabeled 2-methylenacyl-CoA thioesters (**12a–c**) were designed as AMACR binding ligands. The thioesters were tested for their ability to inhibit the AMACR-mediated epimerization of (25*R*)-THC-CoA and were found to be strong AMACR inhibitors. Radioiodinated (*E*)-$^{131}$I-13-iodo-2-methylentridec-12-enoic acid ($^{131}$I-**7c**) demonstrated preferential retention in AMACR-positive prostate tumor cells (LNCaP, LNCaP C4-2wt and DU145) compared with both AMACR-knockout LNCaP C4-2 AMACR-siRNA and benign BPH1 prostate cell lines. A significant protein-bound radioactive fraction with main bands at 47 (sum of molecular weights of AMACR plus **12c**), 70, and 75 kDa was detected in LNCaP C4-2 wt cells. In contrast, only negligible amounts of protein-bound radioactivity were found in LNCaP C4-2 AMACR-siRNA cells.

**Keywords:** antitumor agents · fatty acids · imaging agents · inhibitors · metabolism · radiopharmaceuticals

## Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed cancers and the second leading cause of cancer-related death among men in the western industrialized nations. In the United States of America, about 27360 deaths have been estimated to result from PCa in 2009.[1] $^{18}$F-2-Deoxy-2-fluoroglucose (FDG), which is an indicator of glycolytic activity in cancer cells, is generally ineffective in the diagnosis of localized PCa due to the low metabolic glucose activity of PCa compared with other cancer types.[2] Numerous studies revealed that PCa is associated with changes to fatty acid metabolism. Therefore, $^{11}$C-choline, $^{18}$F-fluoromethyl-, or $^{18}$F-fluoroethylcholine, which target upregulated lipid synthesis, have been employed in molecular imaging of PCa.[3] However, normal and hyperplastic prostatic tissues may also accumulate choline-derived tracers, that consequently leads to false positive diagnoses.[4,5]

α-Methylacyl-CoA racemase (AMACR) is a peroxisomal and mitochondrial enzyme involved in β-oxidation of α-branched fatty acids that catalyses the interconversion of (*S*)/(*R*)-isomers of a range of α-methylacyl-CoA thioesters (Scheme 1).[6,7] AMACR is overexpressed in various cancers such as colon, pulmonary, renal, and liver cancers.[8–10] In PCa, AMACR expression level is high in more than 95% of cases, whereas more than 99% of normal and benign prostate tissues demonstrate either no or only low AMACR expression.[11–13] Furthermore, inhibition of AMACR expression was shown to induce the reversion of androgen dependency in advanced prostate cancer, which may present a new treatment option for hormone-refractory prostate cancer.[14] However, in spite of the potential of AMACR as an anti-

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[**] AMACR = α-Methyl-CoA-racemase.

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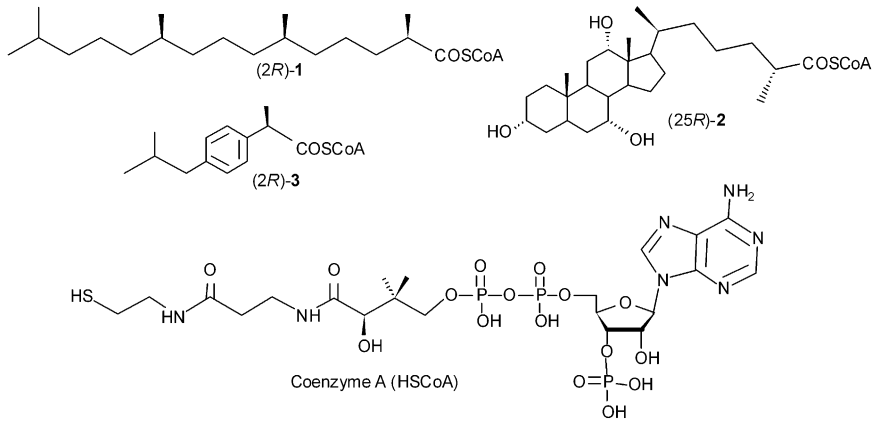


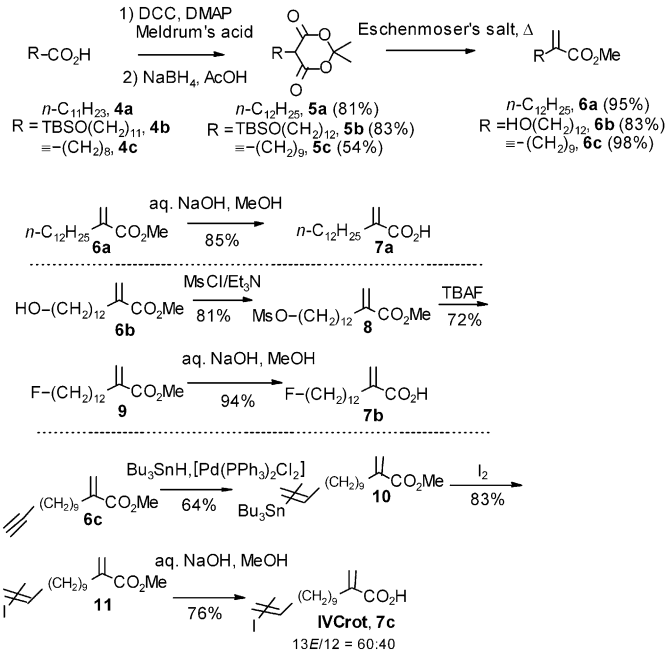

Scheme 1. Structures of some substrates of AMACR: pristanoyl-CoA (**1**), 3α,7α,12α-trihydroxycholestanoyl-CoA (THC-CoA; **2**), ibuprofenyl-CoA (**3**), and coenzyme A. AMACR = α-Methyl-CoA-racemase.

cancer target, the development of high-affinity AMACR-specific substances has so far attracted little attention.[15] Not surprisingly, no AMACR-selective markers for molecular imaging have been published so far. Here, we present the synthesis and in vitro evaluation of new AMACR-inhibitors as potential molecular tracers for PCa and other AMACR-positive tumors.

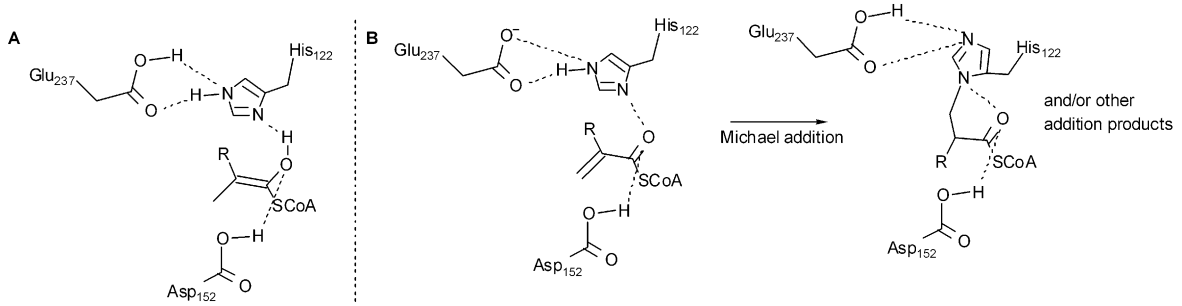
## Results and Discussion

As demonstrated[16,17] for *M. tuberculosis* and human homologues, AMACR catalyses the chiral inversion of (2*R*)- and (2*S*)-2-methylacyl-CoA through a 1,1-proton transfer mechanism. 2-Methylacyl-CoA undergoes deprotonation and forms a planar enolate with $His^{122}$ (number refer to human AMACR IA) acting as a base to deprotonate the substrate, followed by addition of $H^+$ with $Asp^{152}$ as a proton donor (Scheme 2A). $Glu^{237}$ is also involved in epimerization. AMACR exhibits broad substrate selectivity (Scheme 1). However, all substrates and inhibitors of AMACR known to date are 2-methyl- or 2-difluoromethyl- and 2-trifluoromethylacyl-CoA thioesters. We envisaged that the substrate selectivity of AMACR could be broader than assumed, and that 2-methylenacyl-CoA substrates could also fit the catalytic pocket. In this case, the substrates may undergo Michael addition with the imidazole ring of the active site base or another nucleophilic group to give covalent adducts with the enzyme (Scheme 2B).

To investigate this approach, we prepared three 2-methylenacyl-CoA compounds, including those with fluorine or iodine substituents. The synthesis commenced with acylation of Meldrum's acid with the corresponding carboxylic acid **4a–c**, followed by reduction with $NaBH_4$ to give monosubstituted Meldrum's acids **5a–c** (Scheme 3) by using a previously reported method.[18] The latter compounds were treated with Eschenmoser's iodide salt according to the published protocol[19] to give α-substituted methyl acrylates **6a–c** in




Scheme 3. Synthesis of **7a–c**. Eschenmoser's salt = *N*,*N*-dimethylmethylenammonium iodide; MsCl = methanesulfonyl chloride.



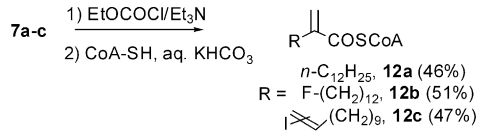

Scheme 2. A) Proposed[16,17] intermediate for the AMACR-catalyzed epimerization. B) Hypothetical mechanisms of possible AMACR inhibition by 2-methylenacyl-CoA thioesters.

52–87% over three steps. It is worth noting that concomitant quantitative deprotection of the *O*-TBS group was observed in the case of **5b**.

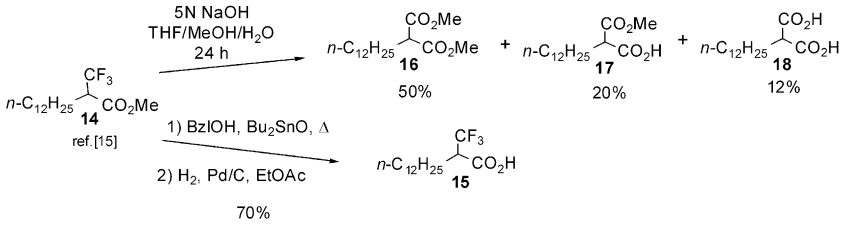
2-Methylenmyristic acid (**7a**)[20] was obtained by base-mediated hydrolysis of the respective ester **6a** in 85% yield (Scheme 3). 14-Fluoro-2-methylentetradecanoic acid (**7b**) was prepared from **6b** in 55% yield over three steps by mesylation, followed by treatment with tetrabutylammonium fluoride (TBAF) and, finally, base-mediated hydrolysis of the intermediate ester **9**. Compound **6c** was hydrostannylated according to Zhang et al.,[21] followed by iodination with $I_2$ to give, after base-mediated hydrolysis, iodo-2-methylentridecen-12-oic acid (IVCrot, **7c**) as a mixture of regioisomers (13*E*/12 = 60:40) in 40% yield over three steps.

Reaction of coenzyme A with the mixed anhydrides of acrylic acids **7a–c** and ethyl chloroformate gave CoA-thioesters **12a–c** in 46–51% yield and more than 90% purity (HPLC) (Scheme 4).




Scheme 4. Synthesis of **12a–c**. CoA-SH = coenzyme A.

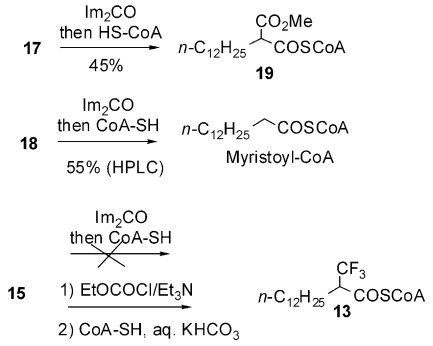
2-Trifluoromethylmyristoyl-CoA (**13**), which is the most effective AMACR inhibitor described to date, inhibits epimerization of **2** in low micromolar range.[15] We decided to synthesize **13** to compare its AMACR inhibition activity with those of **12a–c** (Scheme 4). Unexpectedly, base-mediated hydrolysis of methyl 2-trifluoromyristinate (**14**) under the described conditions[15] gave, instead of acid **15**, a mixture of dimethyl- and monomethyl dodecyl malonates as well as dodecylmalonic acid (**16–18**) (Scheme 5).




Scheme 5. Products of the base-mediated hydrolysis of **14** and synthesis of **15**.

The dependence of the base-mediated hydrolysis of **14** on the concentration of NaOH (0.5–10 M), reaction time, and temperature (0–40 °C) was studied. Under none of the tested conditions even traces of **14** or **15** were found in the reaction mixture after addition of more than two equivalents of NaOH.[22] Consequently, we prepared an authentic sample of **15** by transesterification of **14** with benzyl alcohol using $Bu_2SnO$ as catalyst,[23] followed by hydrogenolysis of the intermediate benzyl ester in 70% yield over two steps. In our hands, it was not possible to synthesize acid **15** according to the published procedure for its preparation.[15] Instead, compounds **16–18** were obtained. Therefore, the CoA-thioester of **17** or **18** and not **13** was prepared and shown to be a strong AMACR inhibitor. Consequently, we decided to synthesize these compounds and test their AMACR inhibitory activity.

Treatment of **17** with $Im_2CO$, followed by reaction of the intermediate acylimidazolide with CoA-SH as described for preparation of **13**,[15] gave the desired product **19** in 45% isolated yield (Scheme 6). However, attempts to prepare do-




Scheme 6. Preparation of **13** and **19** as well as myristoyl-CoA.

decylmalonyl-CoA (**20**)[24] by the same route gave myristoyl-CoA in 55% yield (HPLC) as the only detectable product of thioesterification, presumably due to extensive decarboxylation of the intermediate monoimidazolide. Compound **13** could be obtained, albeit in moderate yield, from **15** via the mixed anhydride but, again, not from the acylimidazolide.

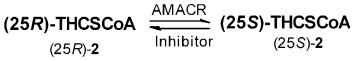
Inhibition of the AMACR-catalyzed epimerization of (25*R*)-THC-CoA [(25*R*)-**2**][15,25] by 2-substituted acryloyl-CoAs **12a–c** was studied to evaluate the potential of the newly prepared compounds as AMACR inhibitors. Gratifyingly, thioesters **12a–c** were capable of inhibiting the epimerization of (25*R*)-**2** up to 80–98%, compared with approximately 60% inhibition by 2-methylmyristoyl-CoA,[7,26] which was used as a control (Table 1). Compound **13** also exhibited strong AMACR inhibition. In contrast, neither **19** nor myristoyl-CoA significantly inhibited α-methylacyl-CoA-racemase.

Because the presence of a metabolically stable iodovinyl group allows switching between SPECT, PET, and radiotherapy agents simply by using different iodine radioisotopes ($^{123}I$, $^{124}I$ and $^{131}I$, or $^{125}I$, respectively) with conveniently long half-life times that permit prolonged biological studies, IVCrot (**7c**) and its CoA-thioester **12c** were chosen for further study.

A kinetic study of AMACR inhibition by IVCrot-CoA (**12c**) was carried out using different concentrations of inhibitor **12c** (0–300 μM) and the substrate (25*R*)-**2** (15–75 μM)

Table 1. Study of AMACR inhibition.




| Inhibitor | Epim. [%][a–d] |
|---|---|
| – | 100 ± 1.8 (3) |
| 2-methylmyristoyl-CoA | 40.8 ± 9.9 (4) |
| **12a** | < 2 (3) |
| **12b** | < 5 (3) |
| **12c** | 20.1 ± 1.8 (3) |
| **13** | < 2 (3) |
| **19** | 91.7 ± 4.3 (3) |
| myristoyl-CoA | 91.7 ± 2.7 (3) |

[a] Assay conditions: 60 μM (25*R*)-**2**, 200 μM inhibitor, 14.5 μg mL$^{-1}$ rat liver homogenate was used as the AMACR source, 50 mM Na/K phosphate buffer pH 7.22, 35 °C, 15 min.; [b] (25*R*)/(25*S*)-**2** Ratio was determined by HPLC analysis. [c] Epimerization in the absence of inhibitor was taken as 100 %. [d] The number of runs is given in parentheses.

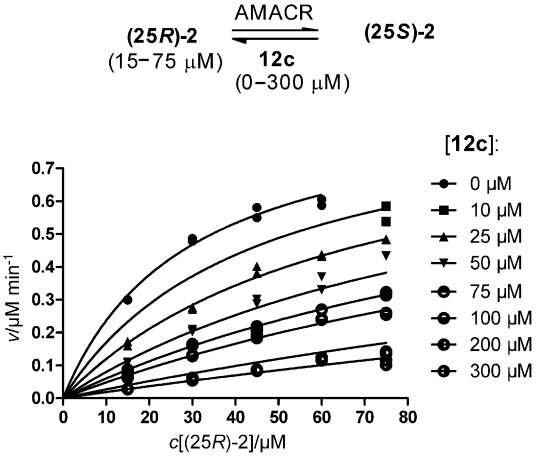
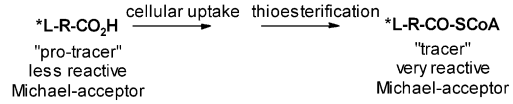



Figure 1. Kinetic plot for inhibitor **12c**.

(Figure 1). Fitting the data to competitive, uncompetitive and non-competitive inhibition models using GraphPad Prism[27] and selecting the model most consistent with the data using the corrected Akaike information criterion,[28] showed that the kinetics were best described by competitive enzyme inhibition; this process yielded the following parameters: Michaelis–Menten constant $K_M = 29.0 \pm 2.8$ μM, maximum enzyme velocity $v_{max} = 0.919 \pm 0.028$ μM min$^{-1}$, and inhibition constant $K_i = 19.0 \pm 1.2$ μM (Figure 1).

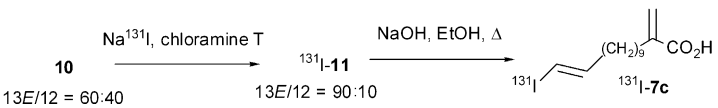
High affinity of the tracer to the target is a prerequisite for successful radiological imaging. However, for metabolic pathway tracers that work on the principle of "metabolic trapping"[29] (FDG, FLT and other nucleosides, methionine, FDOPA, and other amino acids), this parameter is not as important as for receptor targeting tracers (DOTA-TOC, bombesine conjugates, and other peptide tracers). For example, $^{18}$F-FDG enters the cell with the assistance of glucose transporter proteins (mainly GLUT I and Glut III) and is phosphorylated by hexokinase II to give $^{18}$F-FDG-6-phosphate, which cannot be further metabolized due to the fluorine substituent in the 2-position. The high polarity of $^{18}$F-FDG-6-phosphate prevents its passive diffusion out of the cell. Instead, $^{18}$F-FDG-6-phosphate accumulates ("traps") in cells with high sugar metabolism and high GLUTI and HexII levels, although affinity of FDG to HEXII is not very good and, consequently, the respective phosphorylation constants are very high (normally more than 100 μM, often more than 300 μM depending on the cell type).[30] Nevertheless, FDG is the most popular PET-tracer, and this compound works very well in the majority of cases. We proposed that, similar to FDG, **7c** would also be metabolically trapped within the cell. Compound **7c** would be taken up into the cell by fatty acid transporters, where it is thioesterified by long-chain acyl-CoA thioester synthetases (LACS)[31] or xenobiotic-CoA ester ligases,[32] both of which have very broad substrate specificity, to give thioester **12c**,[33] which is a reactive Michael-acceptor and, at the same time, can fit the catalytic pocket of AMACR (Scheme 7). Consequently,




Scheme 7. Proposed concept of substituted acrylic acids as "pro-tracers".

radioactive agents can be trapped in the AMACR-positive cells through covalent binding with AMACR. In AMACR-negative cells, thioester **12c** (except of a relatively small proportion that would be incorporated into the cellular lipid fraction) would be partially hydrolyzed into the free acid, which will leave the cell through passive diffusion and/or efflux. Alternatively, **12c** could be metabolized to give low molecular weight metabolites (i.e., adduct with glutathione), which will leave the cell. Therefore, radioiodinated **7c** was prepared and tested in vitro.

Tin precursor **10** was radioiodinated using chloramine T (CAT) as an oxidant to give, after base-mediated hydrolysis in EtOH at reflux and purification by HPLC, no-carrier-added (n.c.a.) $^{131}$I-**7c** as a single (13*E*)-regioisomer in 56 % radiochemical yield over two steps and in more than 98 % radiochemical purity (Scheme 8).[34]

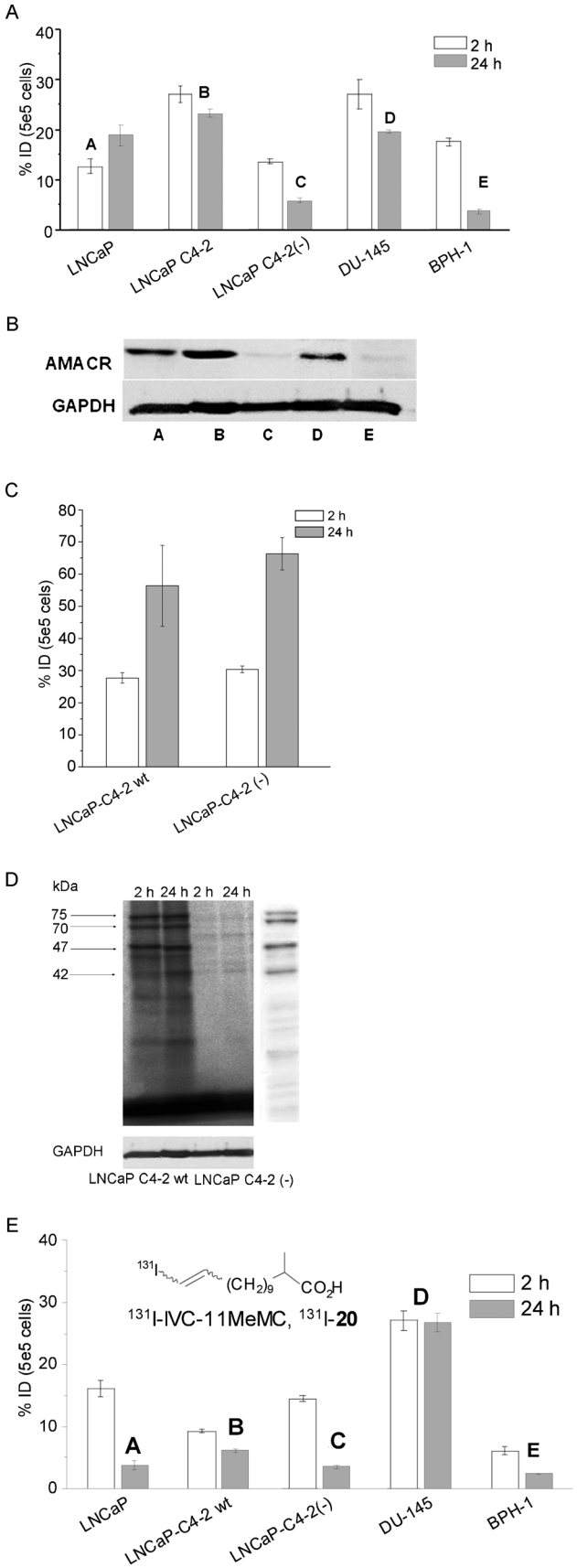



Scheme 8. Radiosynthesis of $^{131}$I-**7c**.

The cellular uptake of $^{131}$I-**7c** was studied in AMACR-positive LNCaP, LNCaP C4-2, and DU-145 prostate tumor cell lines. As a negative control, the benign hyperplasia prostate cell line BPH-1 and siRNA-AMACR knockout LNCaP C4-2 cell line [LNCaP C4-2(–)], which was specially generated for this study, were used (Figure 2 A).[35]

Incubation for 2 h resulted in only moderate selectivity for radioactivity uptake and accumulation in the LNCaP C4-2 wt cell line compared with the LNCaP C4-2 AMACR-




siRNA cell line [percent injected dose (ID %) per $5{\cdot}10^5$ cells: $27.0 \pm 1.5$ vs. $13.6 \pm 0.4$]. Moreover, no significant differences between AMACR-positive malignant and AMACR-negative benign cells were observed (ID %/$5{\cdot}10^5$ cells: 12.7–27.1 vs. $17.5 \pm 0.9$). However, after 24 h incubation, the radioactivity retention in LNCaP C4-2 wt cells was more than four times higher as those in AMACR knockout cells ($23.3 \pm 0.9$ % vs. $5.7 \pm 0.1$ %). Importantly, at this time point, the accumulation of radioactivity was significantly lower in benign BPH-1 than in AMACR-positive tumor cells (ID %/$5{\cdot}10^5$ cells: $3.7 \pm 0.4$ vs. 13.1–23.2) (Figure 2A) and correlated with the expression level of AMACR as determined by western blot analysis (Figure 2B). In contrast, the cellular uptake and accumulation of 1-$^{14}$C-palmitic acid was virtually the same in both, wt and AMACR-siRNA, LNCaP cell lines, indicating that the knockout of AMACR does not affect fatty acid metabolism in general (Figure 2C). The protein-bound radioactivity in lysates obtained from LNCaP C4-2 wt and LNCaP C4-2 AMACR-siRNA cells after incubation with the inhibitor was studied using SDS-gel electrophoresis, followed by phosphorimager analysis (Figure 2D). In contrast to AMACR knockout LNCaP C4-2 cells, a significant protein-bound radioactivity fraction with a distinct band at about 47 kDa (corresponding to the molecular weight of full length AMACR-IVCrot-CoA conjugate) was detected in LNCaP C4-2 wt cells. Interestingly, two additional radioactive fractions with increased molecular weights of approximately 70 and 75 kDa were visualized. This increase in mass from the original 47 kDa is likely due to the dimerization of full length AMACR,[36] which is reported to be the most abundant IA transcript, with its alternatively spliced variants IB (22 kDa) and IIA (28 kDa).[37] Our assumption is supported by the presence of bands with the same molecular weights in the Western blot analysis of AMACR expression in LNCaP C4-2 cells using a polyclonal AMACR-specific antibody.

The radiolabeled precursor of the AMACR substrate, $^{131}$I-(*E*/*Z*)-13-iodo-2-methyltridec-12-enoic acid ($^{131}$I-IVC-11MeMC; $^{131}$I-**20**),[26] which differs from **7c** only by the pres-

Figure 2. Panel A: In vitro study with $^{131}$I-**7c**. Cellular uptake of n.c.a $^{131}$I-**7c** (0.5 MBq + 3 μg FFA/well) in LNCaP (**A**), LNCaP C4-2 wt (**B**), LNCaP C4-2 AMACR-siRNA (**C**), DU-145 (**D**), and BPH-1 (**E**) cell lines. Panel B: Western blot analysis of AMACR expression using monoclonal antibody. GAPDH was used as a loading control. Panel C: Cellular uptake of n.c.a 1-$^{14}$C-palmitic acid (20 kBq + 3 μg FFA/well) in LNCaP C4-2 wt and LNCaP C4-2 AMACR-siRNA cell lines. Panel D: SDS-gel electrophoresis of cell lysates (20 μg protein per lane) obtained from LNCaP C4-2 wt and LNCaP C4-2 AMACR-siRNA cell lines incubated with $^{131}$I-**7c** and visualized by phosphorimager (left) compared with Western blot analysis of AMACR expression in LNCaP C4-2 cells using polyclonal antibody (right). GAPDH was used as a loading control. Panel E: Cellular uptake of n.c.a $^{131}$I-(*E*/*Z*)-13-iodo-2-methyltridec-12-enoic acid ($^{131}$I-IVC-11MeMC, $^{131}$I-**20**) (0.5 MBq + 3 μg FFA/well) in cell lines **1**–**5**. Error bars represent standard deviation (±SD) for three experiments. ID % (5e5) = % of injected dose per $5 \times 10^5$ cells; LNCaP C4-2(−) = LNCaP C4-2 AMACR-siRNA; n.c.a = no carrier added; FFA = fatty acid free albumin, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, SDS-gel electrophoresis = sodium dodecylsulfate polyacrylamide gel electrophoresis.

ence of a methyl instead of methylene group in the second position, was prepared and tested in the same panel of cell lines. Similarly, after 24 h incubation, a higher cellular uptake was observed in LNCaP C4-2 wt cell line than in the AMACR knockout cell line. The intracellular accumulation of $^{131}$I-IVC-11MeMC was higher in AMACR-expressing tumor cells than in benign AMACR-negative BPH-1 cells. However, this effect was generally much less pronounced as for inhibitor **7c** (ID %/5·10$^5$ cells: $6.1\pm0.3$ vs. $3.5\pm0.2$ and 3.9–26.8 vs. $2.4\pm0.1$, respectively). The relatively high cellular uptake and retention of $^{131}$I-**20** in DU145 cells could be explained by factors that are independent of AMACR expression level, for example, by the higher efficiency of the CoA-thioesterification of **20** compared with **7c**[38] and/or by the known high level of expression of acyl-CoA synthase(s) in this cell line (acyl-CoA thioesters are polar and cannot be externalized from the cells).[39]

## Conclusion

In this work, we demonstrated that 2-methylene-substituted acyl-CoA compounds are strong inhibitors of AMACR. The in vitro study with radiolabeled IVCrot ($^{131}$I-**7c**) showed significant AMACR-specific accumulation of radioactivity in prostate tumor cells. Moreover, after incubation with $^{131}$I-**7c**, covalent binding of radioactivity to the intracellular AMACR enzyme was observed in AMACR-positive cells. Because AMACR overexpression starts early in the oncogenesis and persists during progression to higher stages of PCa,[40] IVCrot is a potential candidate for imaging of prostate tumors. Furthermore, IVCrot and other 2-methylene-substituted fatty acids may also be used for the selective inhibition of α-methylacyl-CoA racemase, which can potentially be a new option for treatment of the hormone-relapsed prostate tumors.

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