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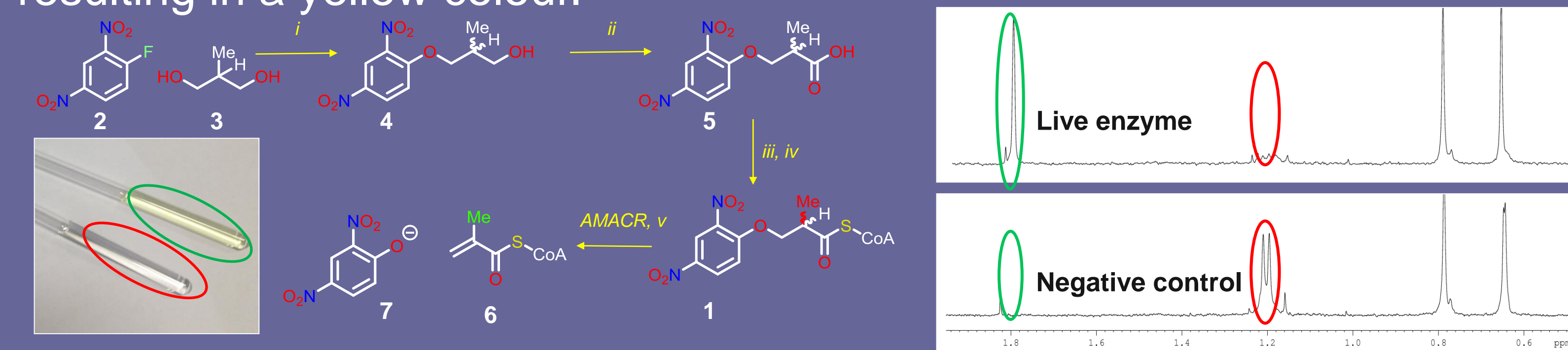
## Introduction

Branched-chain fatty acids are common in the diet and similar structures are found in medicines such as Ibuprofen and related drugs. Metabolism of branched-chain fatty acids requires that the centres bearing the methyl groups possess *S*-stereochemical configuration, but those with *R*-configuration are produced in the body and are found in the diet. Ibuprofen and related drugs require *S*-configuration for their anti-inflammatory properties, but these drugs are usually given as a mixture of *R*- and *S*-enantiomers. The enzyme  $\alpha$ -methylacyl-CoA racemase (AMACR) catalyses *R*- to *S*- conversion of 2-methylacyl-CoA derivatives of fatty acids enabling  $\beta$ -oxidation. Similarly, acyl-CoA derivatives of Ibuprofen and similar drugs are converted, resulting in pharmacological activation.<sup>1,2</sup>

AMACR levels are increased in all prostate cancers, some colon cancers and other cancers.<sup>1-3</sup> In prostate cancer, higher AMACR levels result in higher proliferation rates<sup>4</sup> and androgen-independent growth<sup>5</sup> and AMACR is recognised as a novel drug target. However, few inhibitors have been identified, largely due to the difficulties in measuring enzyme activity which makes it difficult to quantify drug potency.<sup>1</sup> AMACR catalyses the irreversible elimination of hydrogen fluoride from 3-fluoro-2-methylacyl-CoA substrates,<sup>6</sup> but translating this reaction to a convenient colorimetric or fluorometric assay has proven difficult.<sup>3</sup> 4-Nitrophenol derivatives are commonly used as colorimetric substrates for enzymes. This study reports the synthesis of a 2,4-dinitrophenol-containing AMACR substrate and the characterisation of known AMACR inhibitors using a convenient colorimetric microtitre plate assay.

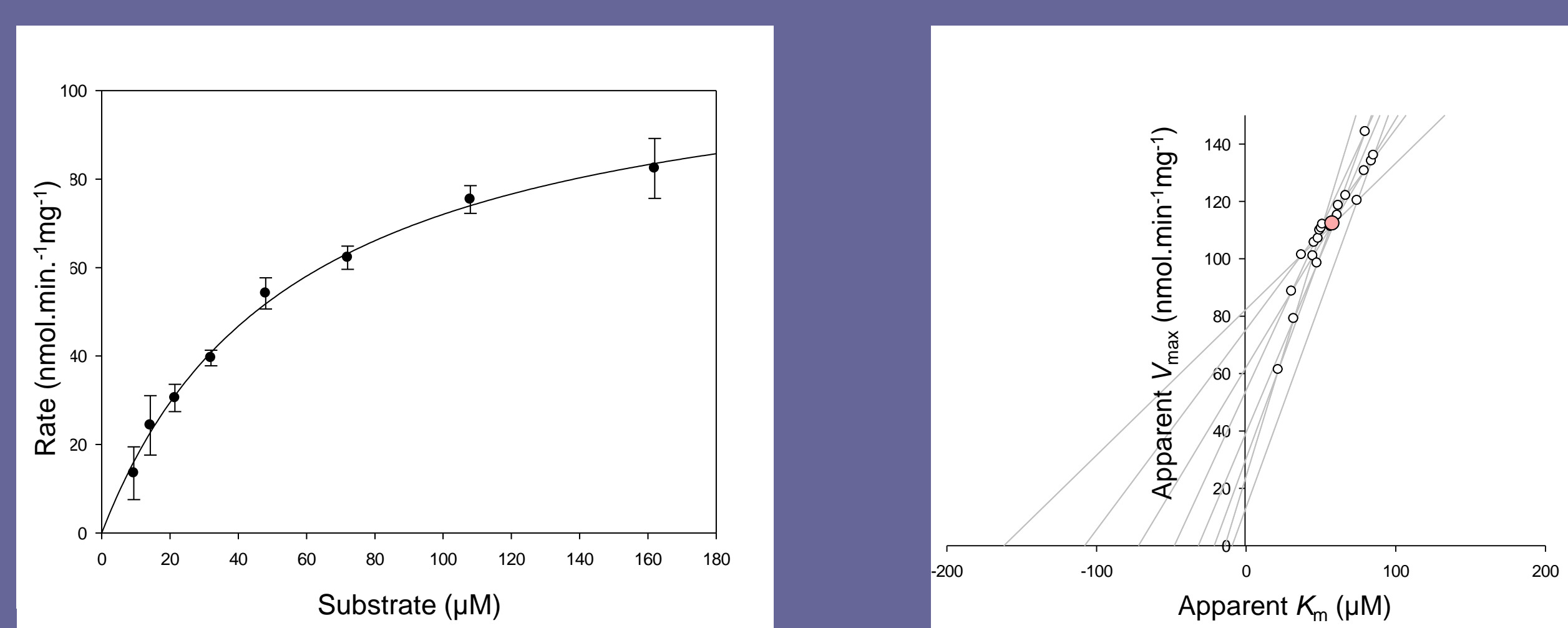
## Results and Discussion

2,4-Dinitrophenol is fully ionised at neutral pH giving a yellow colour and has a similar *pKa* to HF, which is eliminated from known AMACR substrates. Therefore an acyl-CoA derivative **1** containing 2,4-dinitrophenol was investigated. Reaction of **2** with alcohol **3** to give **4** followed by oxidation gave the racemic acid **5**, which was converted to the desired substrate **1** (Scheme 1). Incubation of **1** with recombinant human AMACR 1A resulted in formation of unsaturated product **6** and 2,4-dinitrophenol **7** resulting in a yellow colour.



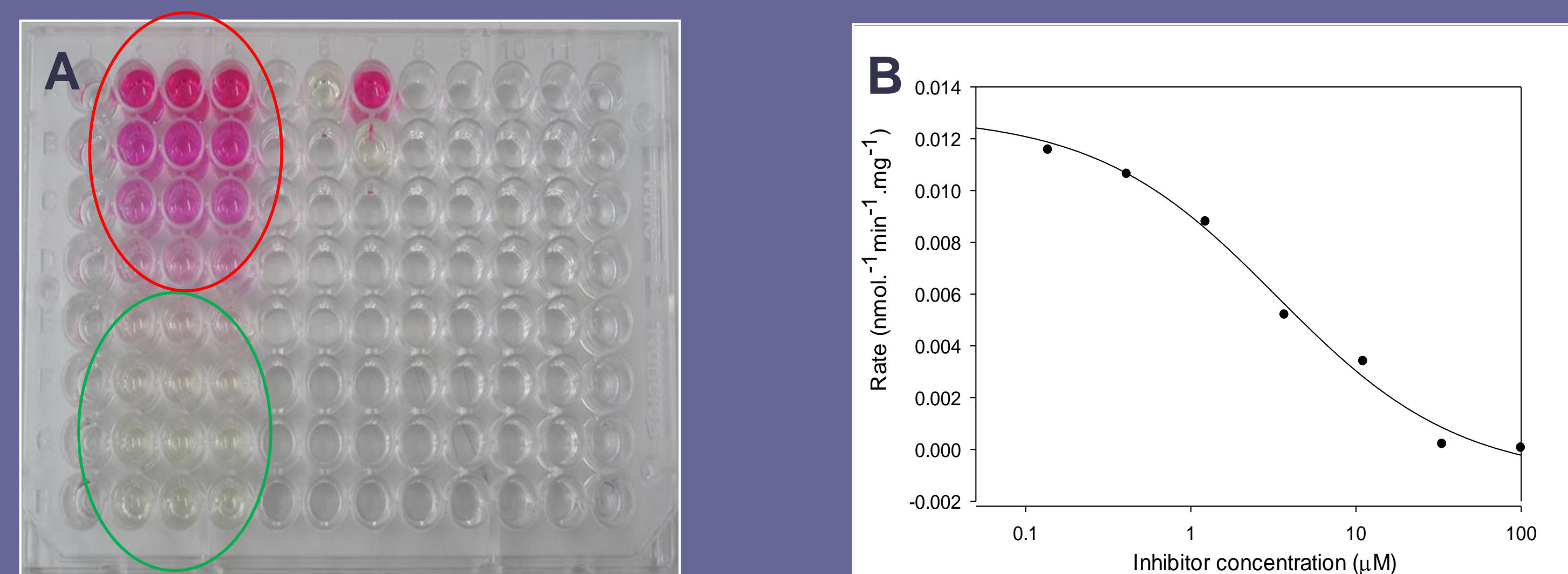
**Scheme 1:** Synthesis of novel substrate **1** and reaction with AMACR. *Reagents & conditions:* i. Na metal; ii. Jones oxidation; iii. CDI, DCM; iv. CoA-SH, NaHCO<sub>3</sub> aq./THF (1:1); v. NaH<sub>2</sub>PO<sub>4</sub>-NaOH, pH 7.4, ca. 77% <sup>2</sup>H<sub>2</sub>O.

AMACR was active around neutral pH and retained full activity in the presence of 8% (v/v) DMSO. Kinetic analysis of substrate **1** showed that Michaelis-Menten kinetics were observed (Figure 1), with the following parameters:  $K_m = 56 \pm 4.5 \mu\text{M}$ ;  $V_{max} = 112 \pm 4 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ;  $k_{cat} = 0.088 \text{ s}^{-1}$ ;  $k_{cat}/K_m = 1571 \text{ s}^{-1} \text{ M}^{-1}$ . This shows that substrate **1** is converted with ~44% of the efficiency of 3-fluoro-2-methyldecanoyl-CoA and was significantly more efficient than 'racemisation' of 2-methyldecanoyl-CoA (as judged by  $k_{cat}/K_m$ ).<sup>6</sup>



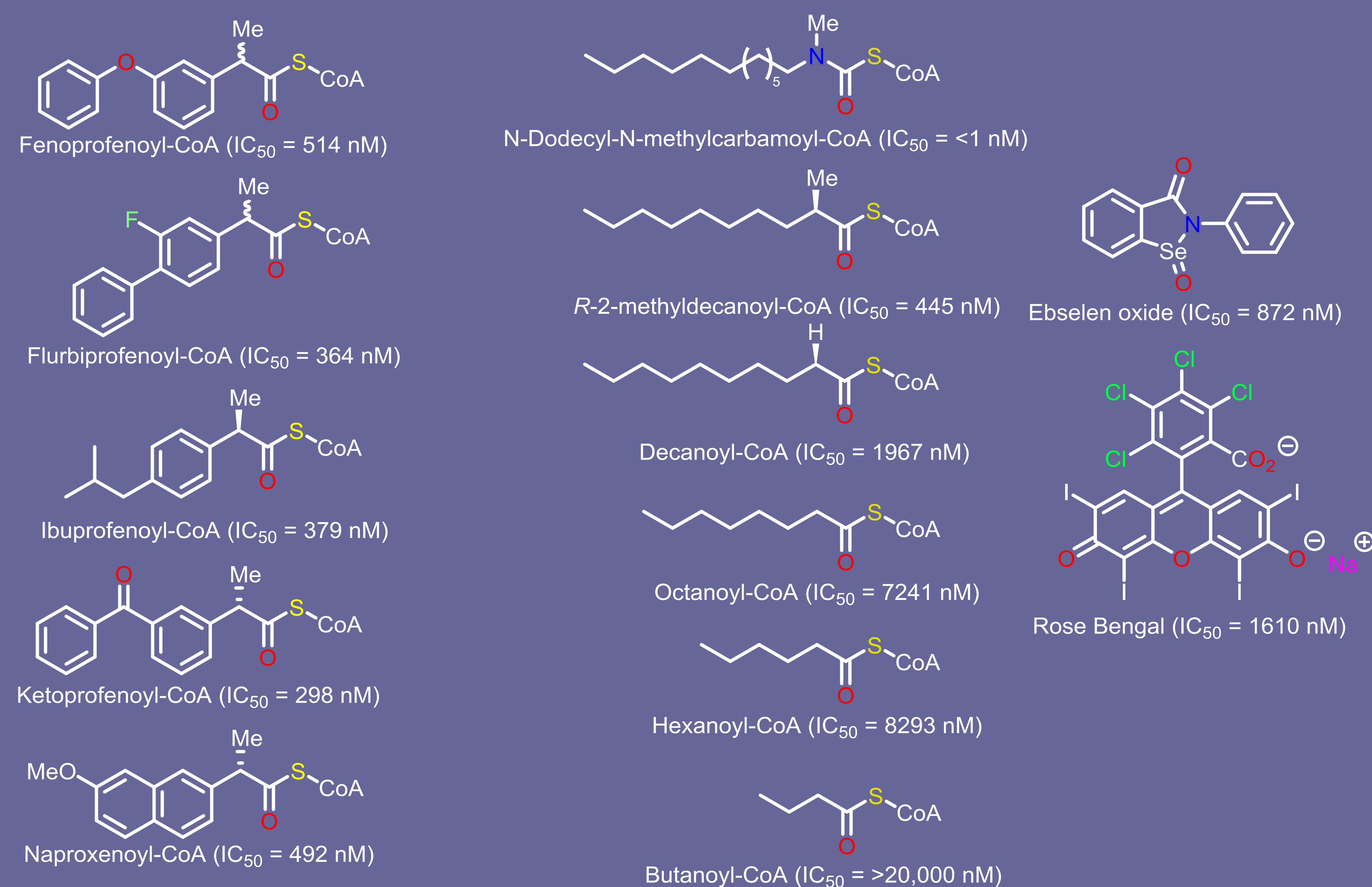
**Figure 1:** Kinetic analysis for substrate **1**.

The known inhibitor Rose Bengal<sup>7</sup> was tested to validate the method for characterisation of inhibitors (Figure 2). A dose-response curve was efficiently produced using a microtitre plate assay.



**Figure 2:** AMACR inhibition assay using Rose Bengal as an inhibitor. A. 96-Well plate showing colour change; B. Dose-response curve for Rose Bengal.

A number of other known AMACR inhibitors and substrates were tested using a dose-response curve at a fixed substrate concentration of 40  $\mu\text{M}$ . Ibuprofenoyl-CoA and related compounds are known substrates and should behave as competitive inhibitors. All of these compounds inhibited the enzyme with  $\text{IC}_{50}$  values of ca. 300-500 nM. 2-Methyldecanoyl-CoA also inhibited the reaction, and was ca. 4x more potent than decanoyl-CoA. Inhibition was decreased in acyl-CoA esters with shorter alkyl chains. The best acyl-CoA inhibitor was *N*-dodecyl-*N*-methylcarbamoyl-CoA,<sup>8</sup> which was ~500 – 1000 x more potent than the other acyl-CoA inhibitors (as judged by  $\text{IC}_{50}$  values). The non-specific protein modifying reagents reported by Wilson *et al.*<sup>7</sup> also inhibited the enzyme; in contrast to previous reports Ebselen behaved as a time- and concentration-dependent inactivator with a rate constant of 114  $\text{M}^{-1} \text{ s}^{-1}$ .



**Figure 3:** Selected acyl-CoAs and protein modifying agents shown to inhibit the conversion of substrate **1** to **6** and **7** by AMACR using the colorimetric assay.

## Conclusions

The colorimetric substrate **1** provides a convenient method for assaying AMACR and determining the behaviour and potency of inhibitors. AMACR is a promising drug target for prostate and other cancers, but until now it has been under-exploited because of the difficulties in determining enzyme activities. Inhibitors previously reported in the literature are largely limited to rationally designed acyl-CoA esters, which do not comply with Lipinski guidelines.<sup>9</sup> This new assay will facilitate the testing and development of drugs by structure-based design, rational design and lends itself to screening approaches. The latter should allow identification of inhibitors with good drug-like properties.

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