Enzyme Promiscuity

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Fluoromorphic Substrates for Fatty Acid Metabolism: Highly Sensitive Probes for Mammalian Medium-Chain Acyl-CoA Dehydrogenase**

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In contrast to the accepted view in biological sciences that enzymes are highly selective for their physiological substrates, substrate promiscuity is a widespread phenomenon.[1] Such functional flexibility may often be observed with enzymes involved in the metabolism of an array of substrates, and examples extend beyond the well-known xenobiotic metabolism.

The exploration of enzyme substrate flexibility for the purpose of the development of metabolic probes and imaging agents represents a guiding concept for a broad program in our laboratories.[2,3] Herein we report the examination of the substrate fidelity of medium-chain acyl-CoA dehydrogenase (MCAD), a key enzyme in the metabolism of fatty acids, which led to the development of fluorogenic and fluoromorphic probes for this enzyme.[4] These indicators allow for selective and sensitive detection of MCAD activity in tissue homogenate.

β-Oxidation of fatty acids represents one of the central metabolic pathways.[5–11] Close examination of this process reveals considerable built-in substrate flexibility. Catabolism of a long-chain fatty acid occurs by repetitive removal of two-carbon units (acetyl-CoA), (Figure 1). Each catalytic turn involves four chemical steps that are catalyzed by enzymes capable of accommodating substrates with a variety of chain lengths. Thus, instead of having a specific enzyme for each intermediate (C_{16}, C_{14}, $C_{12} \rightarrow C_2$), this pathway consists of a few enzymes that show broad and overlapping chain-length specificities.

The overall mechanism for this pathway was proposed on the basis of classical studies conducted by F. Knoop[12] who showed that ω-phenyl fatty acids were metabolized in dogs.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

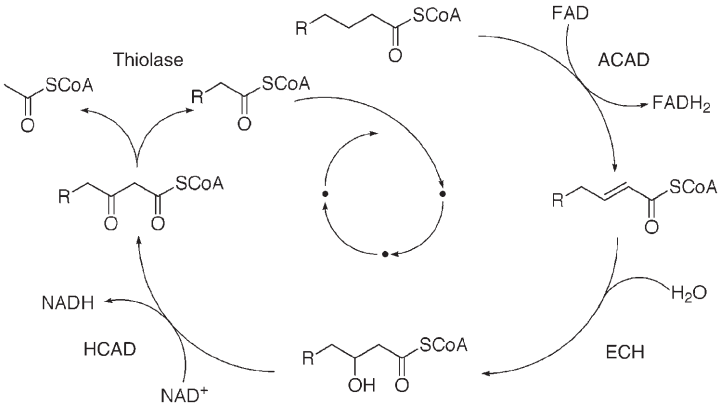


Figure 1. The β-oxidation cycle. The four enzyme families involved in β-oxidation are: acyl-CoA dehydrogenase (ACAD), enoyl-CoA hydratase (ECH), L-3-hydroxyacyl-CoA dehydrogenase (HCAD), and thiolase.

The ingenious use of the phenyl ring as a chemical label suggested a two-carbon degradation pathway but also, from the perspective of this paper, suggested the permissiveness of the entire pathway.[13]

The β-oxidation spiral begins, after the activation (acyl-CoA synthesis) and transport of fatty acids into mitochondria, with the α,β-dehydrogenation of acyl-CoA catalyzed by acyl coenzyme A dehydrogenases (ACADs). This flavin-dependent family consists of nine isozymes, five of which are involved in fatty acid metabolism.[10] Medium-chain acyl-CoA dehydrogenase (MCAD), formerly known as the 'general' ACAD owing to its ability to act on a wide range of chain lengths (C_{16}–C_4, Figure 2), represents an attractive target for the development of reporter substrates.

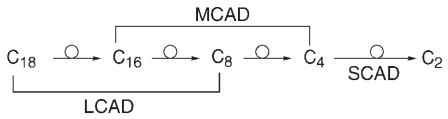


Figure 2. Substrate flexibility of medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD). These enzymes show broad and overlapping chain-length specificities. Acyl-CoAs with eight (C_8) to 18 (C_{18}) carbon chains are substrates for LCAD, whereas those with four (C_4) to 16 (C_{16}) are substrates for MCAD. SCAD shows low flexibility with butanoyl-CoA (C_4) being the main substrate.

MCAD is also an important target from the medical perspective. MCAD deficiency has recently emerged as a common hereditary disease with severe symptoms.[14–17] Furthermore, growing evidence implicates impaired β-oxidation as an important contributor to the development of a cluster of diseases such as hypertension, insulin resistance, and dyslipidemia, which is referred to as 'metabolic syndrome'.[18–20] Consequently, there is a need for a direct, sensitive, and continuous readout of MCAD enzyme activity in tissue samples and cell lines. Inspired by this challenge, we aimed to develop a fluorometric assay that is based on fluorogenic probes.

To develop fluorogenic MCAD enzyme reporters, two key criteria must be satisfied: 1) dehydrogenation is coupled either to an increase in brightness (fluorogenic) or preferably to a significant shift in emission wavelength (fluoromorphic), and 2) these compounds serve as substrates for MCAD.

Our design was based on a general motif that consists of an organic fluorophore attached to the β-carbon of propionyl-CoA (Figure 3). Dehydrogenation provides the corresponding unsaturated system with an extended π-conjugation that connects the fluorophore and the thioester group. We anticipated that such dramatic expansion of the π-conjugated system would strongly affect the emission profile, most likely shifting the emission to longer wavelengths.

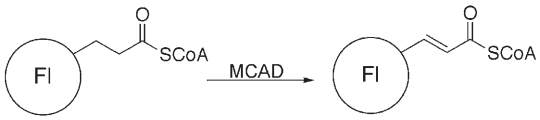


Figure 3. Design of a MCAD fluorescent reporter. Dehydrogenation extends the π-conjugation of the fluorophore, which results in a change in the emission profile. (Fl = organic fluorophore).

It has been shown that Knoop's substrate, 3-phenylpropionyl-CoA, and related compounds are substrates for MCAD; however, neither the substrates nor the products are fluorescent.[21–23] Consequently, we began with naphthalene-based systems, namely compound **1**, the dimethylamino-substituted analogue **2** (Figure 4),[24] and their corresponding products **3** and **4**, respectively. The photophysical measurements revealed that both pairs, **1**/**3** and **2**/**4**, constituted excellent fluoromorphic switches. Between compound **1** and **3**, the emission maximum is red shifted by approximately 50 nm with some overlap between the two curves. A threefold change in the emission intensity could be measured at the emission maximum of each compound, therefore meeting the requirements of a ratiometric probe. A comparison of **2** and **4** revealed a 190-nm shift of the emission and a 100-fold increase in the fluorescence intensity at the emission maximum of compound **4** (Figure 4).[25]

As the emission switch criterion was satisfied, we next addressed the issue of whether compounds **1** and **2** were substrates for MCAD. The activity was tested with purified rat-liver MCAD (rMCAD) by monitoring the reduction of an external electron acceptor (for assay conditions, see Supporting Information). We were gratified to find that compound **1** was a good substrate, however, **2** was completely inert. We hypothesized that the high polarity of the dimethyamino group, not necessarily its size, disfavored the interaction with the hydrophobic pocket of the enzyme. Instructed by these results, we synthesized compounds **5**–**10** to systematically explore two questions: 1) the limits of this enzyme in terms of the size, shape, and polarity of the substrate, and 2) the effect of substituents and other structural variations on emission properties.

Of the series shown in Figure 5, only probes **7** and **9** were substrates for MCAD. The adverse effect of polar substituents was confirmed; compounds **5** and **6** were found to be inert, whereas the conversion of the hydroxy group in **5** into a

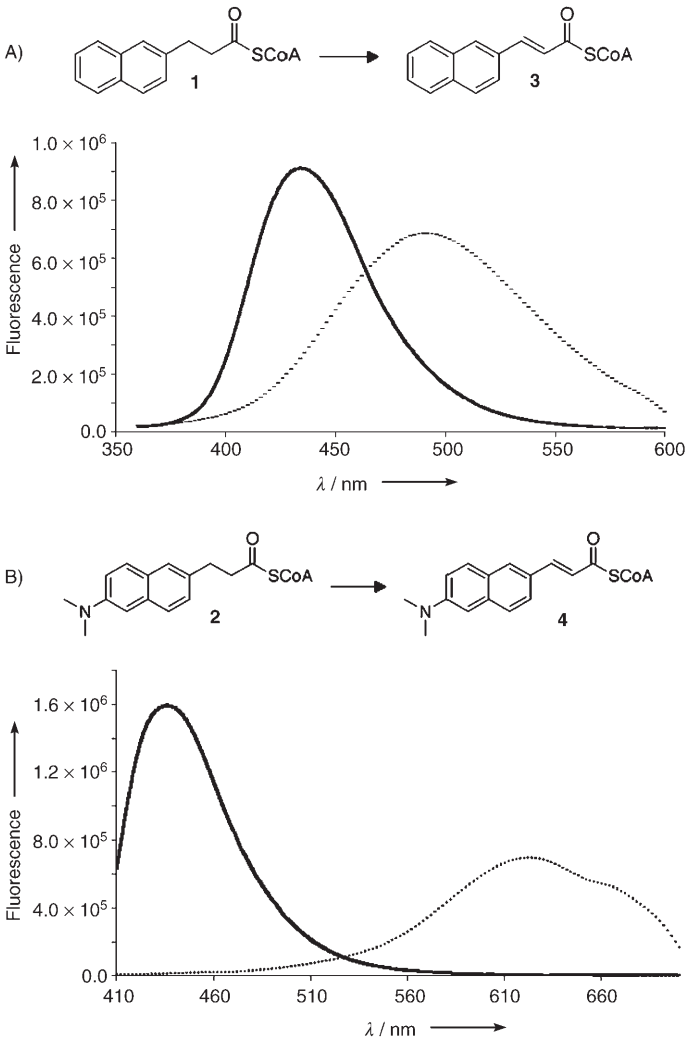


Figure 4. A) Emission spectra of **1** (——) and **3** (-----) ($\lambda_{ex}=340$ nm). B) Emission spectra of **2** (——) and **4** (••••) ($\lambda_{ex}=390$ nm). 50 μM in 100 mM potassium phosphate buffer (pH 8).

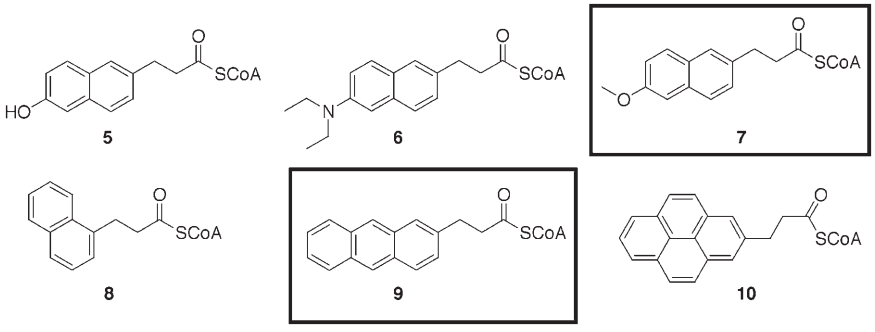


Figure 5. Concurrent exploration of MCAD substrate flexibility and emission properties of fluorophores. Esters **7** and **9** are substrates for MCAD.

methoxy group, as in **7**, furnished a good substrate. The shape of these probes was also of consequence as was demonstrated through the comparison of substrate **1** with its regioisomer **8**. The latter compound, wherein the propionyl chain is attached to C1 instead of C2, was completely inert. This is consistent with structural studies that revealed a narrow active site of MCAD.[10] Interestingly, the attachment of an additional ring to form anthracene was tolerated, demonstrating significant recognition-site flexibility as long as the substrate can assume a narrow shape. The pyrene system **10** was presumably too large and as a result, showed no conversion by the enzyme (Figure 5).

Thus, besides mapping the permissiveness of MCAD, this exercise also identified two new substrates with favorable emission properties (Figure 6). Dehydrogenation of the weakly fluorescent probe **7** led to the highly fluorescent product **11**, which resulted in an 80-fold increase in the emission intensity at 510 nm (fluorogenic probe), whereas conversion of **9** into **12** gave a 150-nm shift in the emission wavelength and afforded an 80-fold increase in emission intensity at 567 nm (fluoromorphic/ratiometric probe). In comparison with probe **1**, better separation of emission curves and a longer emission wavelength was achieved with substrates **7** and **9** (Figure 6).

With three fluorogenic probes in hand, a continuous fluorometric assay was developed and the kinetic parameters were determined for each substrate with rMCAD as well as pig MCAD (pMCAD; Table 1). The fluorometric assay was validated by comparison with the standard spectrophotometric assay (UV/Vis absorption); an excellent agreement was found between these two methods (see Supporting Information). Interestingly, all three probes had K_M^{app} on the same order of magnitude (low micromolar) as the optimum physiological substrate, octanoyl-CoA. Probe **7** had the highest K_M^{app} for both rMCAD and pMCAD, which supports the idea that polar groups disfavor binding to the hydrophobic pocket of the enzyme. In contrast to K_M^{app}, k_{cat} values were significantly lower when compared with octanoyl-CoA (Table 1). Substrate **1** had the largest turnover frequency ($k_{cat}=370\pm10$ and $150\pm4\ min^{-1}$ for rMCAD and pMCAD, respectively); substitution or extension of this motif led to a further decrease in this quantity (Table 1).

We noted that the decrease in enzyme activity ($u\,mg^{-1}$) for each additional phenyl ring in the substrate bears a striking similarity to the chain-length–activity relationship of natural fatty acids (Figure 7). Specifically, 3-phenylpropionyl-CoA had very similar specific activity to the optimal physiological substrate, octanoyl-CoA, whereas probe **1**, which contains the naphthalene system, had a similar activity to dodecanoyl-CoA (C_{12}, lauryl-CoA). Moreover, there was only a fivefold difference in activity between the anthracene probe **9** and palmitoyl-CoA (C_{16}). We propose a simple rationale for these findings based on similarities in the steric demand between the regular fatty acids and synthetic probes. These similarities become apparent when the former substrates are viewed in a folded conformation (Figure 7). X-

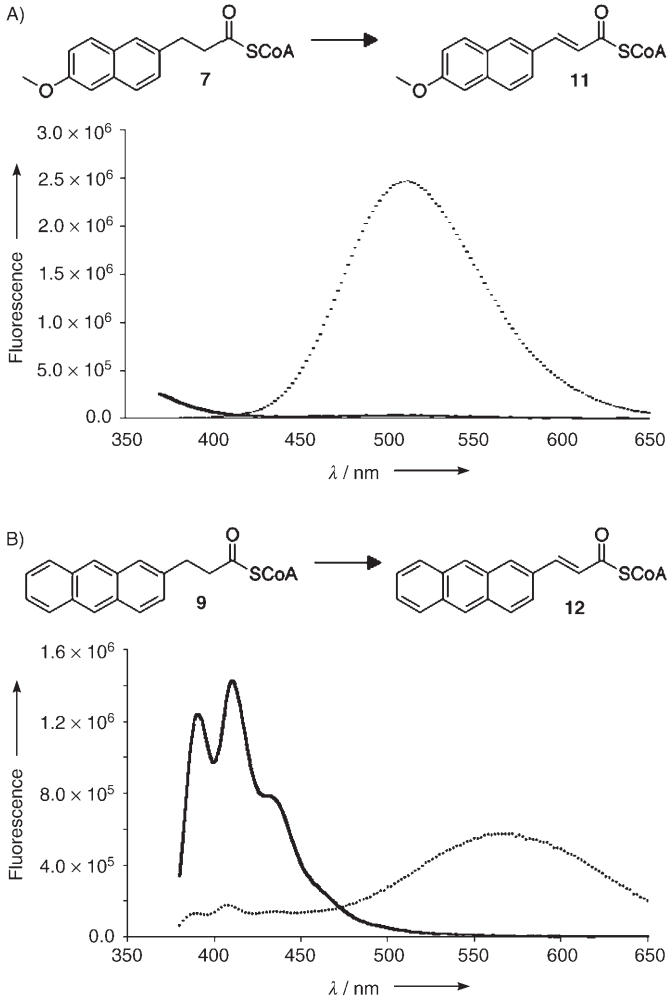
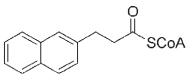
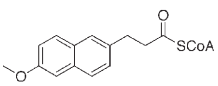
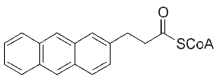


Figure 6. A) Emission spectra of **7** (——) and **11** (-----) (λ_{ex} = 350 nm). B) Emission spectra of **9** (——) and **12** (••••) (λ_{ex} = 356 nm). Compound **7**, **9**, **11**, and **12** (50 μM) in potassium phosphate buffer solution (100 mM, pH 8). The fluorescence is not affected by the assay medium.

Table 1: Kinetic parameters of probes (see Supporting Information).

		K_M^{app} [μM]	k_{cat} [min^{-1}]
octanoyl-CoA	rMCAD[30]	4.0	2142
	pMCAD[31]	2.3 ± 0.1	1176
	rMCAD	2.5 ± 0.2[a]	370 ± 10
	pMCAD	2.3 ± 0.2	150 ± 4
	homogenate	2.2 ± 0.3	–
	rMCAD	6.01 ± 0.7	16.1 ± 0.3
	pMCAD	14.5 ± 1.5	12.3 ± 0.5
	homogenate	3.7 ± 0.4	–
	rMCAD	1.1 ± 0.2	10.4 ± 0.3
	pMCAD	4.5 ± 0.5	13.1 ± 2
	homogenate	1.9 ± 0.4	–

[a] Mean ± S.D. (n = 3).

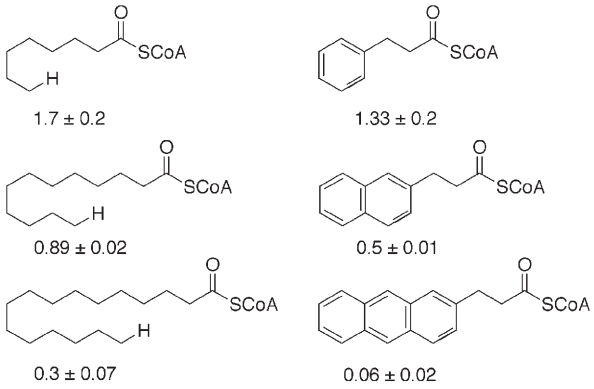


Figure 7. Specific activity (u mg^{-1}) for straight-chain acyl-CoA's and aromatic substrates with rMCAD (see Supporting Information for assay conditions).

Ray crystallographic analysis of the enzyme–substrate complex showed that the active site is not long enough to accommodate long substrates in an extended conformation and, as a result, folding is required (although not to the extent shown in Figure 7).[10,26]

The next major task centered on the evaluation of the possibility of using these substrates as useful probes in tissue samples. This included addressing the issues of selectivity, stability, and the ability to be metabolized by subsequent enzymes of the β-oxidation pathway.

In addition to MCAD, there are two other soluble isozymes of the ACAD family that are involved in the metabolism of straight-chain fatty acids, namely short-chain acyl-CoA dehydrogenase (SCAD) and long-chain acyl-CoA dehydrogenase (LCAD). Purified pig SCAD (pSCAD) and human LCAD (hLCAD) were obtained and tested with probes **1**, **7**, and **9**. Importantly, none of these probes showed any significant activity, which demonstrates a notable selectivity for MCAD within the ACAD family. To address this question in a relevant context, competitive substrate assays were conducted by using rat-liver homogenate (Figure 8). Conversion of the probe by the tissue homogenate was monitored both in the presence and absence of a competitive physiological substrate. Addition of butanoyl-CoA and isovaleryl-CoA, the optimal substrates for SCAD and isovaleryl-CoA dehydrogenase (iVAD), respectively, had no effect, which indicates that probes **1**, **7**, and **9** were not substrates for these enzymes. In contrast, octanoyl-CoA competed with the probes, which led to a significant inhibition of fluorescent product formation. A fivefold excess of octanoyl-CoA completely inhibited dehydrogenation of probe **7** as a result of the weaker binding (higher K_M value) of this reporter substrate. Dehydrogenation of all the probes was inhibited by an excess of palmitoyl-CoA, which was consistent with relatively low K_M as well as low k_{cat} values of this substrate for MCAD (Figure 8). These data, including the experiments with isolated isozymes and tissue homogenates, provided compelling evidence for the high selectivity of the probes **1**, **7**, and **9** for MCAD.

The stability of the fluorescent products **3**, **11**, and **12** was also examined. As these compounds may be metabolized by

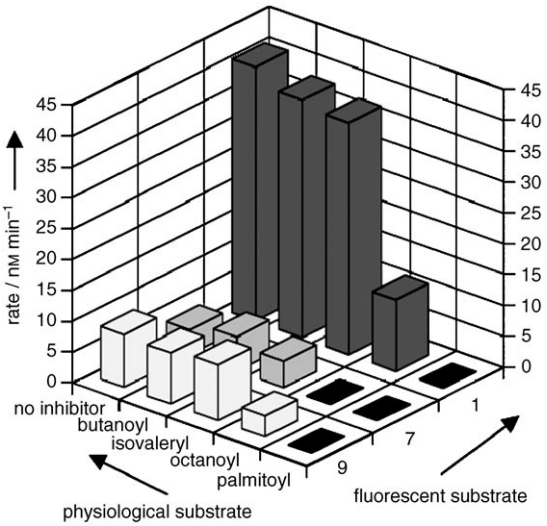


Figure 8. Competitive assays between the fluorogenic substrates (**1**, **7**, and **9**) and the physiological substrates (butanoyl-CoA, isovaleryl-CoA, octanoyl-CoA, and palmitoyl-CoA). Assays were carried out in a 96-well plate with physiological substrate (50 μM), fluorescent substrate (10 μM), and rat-liver homogenate as the enzyme source. The margin of error was $<20\%$ for all assays ($n=3$).

the subsequent enzyme(s) of the β-oxidation pathway, that is, enoyl-CoA hydratase, they were exposed to rat-liver homogenate. Although no hydration occurred, a small decrease in fluorescence was detected with **3** and **11**, which was independent of the homogenate. Rather, products **3** and **11** were subject to photobleaching, whereas compound **12** was stable on the time scale of the experiment. Importantly, photobleaching did not significantly affect the results of the assay.

The relationship between activity and homogenate protein concentration was established for each probe and appears to be linear within narrow ranges of protein concentrations (See Supporting Information). As expected, enzyme activity was dependent on the external electron acceptor, in this case, ferrocenium hexafluorophosphate ($FcPF_6$).[27] When the external oxidant was omitted, probes **1** and **9** were inert, whereas probe **7** gave a detectable residual activity ($\leq 2\%$ of the total activity). As the oxidase activity (direct transfer of electrons to oxygen)[22,23] of the purified MCAD protein was less than 0.03% with probe **7**, the residual activity could be ascribed to either endogenous electron acceptors in the homogenate or peroxisomal acyl-CoA oxidase (ACO).[8] These results indicate that the ACO does not contribute to the observed fluorescent signal in any significant manner, but rather provides further support for high selectivity of these probes for MCAD.

The immediate impact of this study is the ability to monitor MCAD activity in cell and tissue homogenates in a direct and continuous manner, therefore laying the foundation for a new, sensitive, and practical diagnostic test for MCAD deficiency.[28] MCAD deficiency has recently been identified as a common hereditary disease with an estimated occurrence rate of 1:15000 newborns in the US (an incidence rate similar to that of phenylketonuria).[16] This deficiency often results in severe symptoms that resemble those of Reye's syndrome and sudden infant death syndrome. Early diagnosis may prevent the onset of the symptoms through a regulated diet and avoidance of fasting and excessive physical activity.

Generally, fluorometric methods offer superior detection sensitivity (up to two orders of magnitude) in comparison with UV/Vis absorption methods. In this case, the fluorogenic probe **7** allowed for reliable detection of MCAD activity with as little as 0.4 μg of tissue homogenate protein, which is more sensitive than the existing UV/Vis methods.[29] Further to high sensitivity, fluorescence-based measurements may be performed in a high-throughput manner in a routine 96-well-plate format. Based on these results, a new and practical diagnostic test may be developed that offers an attractive alternative to the more involved methods used today (e.g. MS-MS).

In a long-term view, the development of these probes sets the stage for the investigation of the β-oxidation pathway in its intact state. The possibility of measuring and imaging the activity of MCAD, as well as the flux through this pathway, will be examined. This represents an exciting prospect considering the central role of this pathway in a number of metabolic diseases.[17–19]

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