Stable carbon isotope discrimination by human 3-hydroxy-3-methylglutaryl coenzyme A reductase

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Running title: Carbon isotope effect of the HMGR reaction

Abbreviations:
GC-C-IRMS, gas chromatography-combustion-isotope ratio mass spectrometry; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; KIE, kinetic isotope effect; MVL, mevalonolactone; T, testosterone
Abstract

The aim of this study was to investigate the possible existence and magnitude of stable carbon isotope discrimination by human 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). The catalytic portion of HMGR was expressed and purified. The reaction product mevalonate was lactonized and extracted from the reaction mixture by a solid phase extraction protocol. Stable carbon isotope ratios of mevalonolactone (MVL) were analyzed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). An average fractionation factor $^{12}k/^{13}k$ of 1.0031 ± 0.0004 for all carbon atoms contained in MVL was estimated by the method of internal competition. The value was calculated by nonlinear curve fitting, where the ratio $^{13}C/^{12}C$ of MVL was plotted versus the fraction of reaction.

Supplementary key words: mevalonate pathway, steroids, kinetic isotope effect, isotope ratio mass spectrometry, curve fitting, doping control
1 Introduction

Kinetic isotope effects (KIEs) present in enzyme-catalyzed reactions may evidence the corresponding reaction mechanism [1] and account for the natural variation of the stable isotope signatures of biological compounds [2,3]. These parameters are of rapidly increasing interest in the geological, biological, and forensic sciences, to name but some.

The interpretation of such data will be facilitated by a sound understanding of the underlying processes. Although a perfect analysis of isotope fluxes in metabolic networks is virtually impossible [3] and purely empirical “isotope fingerprinting” is applied routinely and successfully, reliable predictors can be found and quantified from time to time. For example, lipids are typically strongly depleted in $^{13}$C versus other classes of biological compounds [4]. This phenomenon usually is attributed to KIEs during the decarboxylation of pyruvate [5].

Considering this phenomenon, the stable carbon isotope ratios of human steroid hormones exhibit somewhat unusual values. Steroids isotopically are only slightly lighter than carbohydrates and proteins [6]. Hence steroid biosynthesis is unlikely to be limited by pyruvate decarboxylation and other processes must control the stable isotope ratios of steroids.

We hypothesized that, due to its key position in steroid biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) might play a pivotal role here. The hypothesis is supported by the observation that steroid hormones reveal stronger $^{13}$C depletion when contraceptives are administered [6]. This probably indicates the emergence of rate limitation resulting from feedback
induced down regulation of the enzyme. HMGR is among the most highly regulated enzymes in nature [7]. The different levels of regulation include transcription, translation, enzyme degradation, and phosphorylation.

HMGR (EC 1.1.1.34) catalyzes the four-electron reduction of HMG-CoA to mevalonate and CoASH: HMG-CoA + 2 NADPH + 2 H⁺ → mevalonate + CoA-SH + 2 NADP⁺. The human enzyme assumes a rate-limiting position in the mevalonate pathway. Numerous biomolecules originate from this process, including haem, ubiquinone, and cholesterol. Besides its diverse functions in animals, the latter forms the backbone of steroid hormones.

Chemical reactions involving bond making or breaking almost always reveal normal isotope effects, which means slower reaction of the isotopically heavier isotopologues [1]. Therefore the reaction of HMGR is likely to discriminate substrate molecules with carbonyl-¹³C vicinal to the thioester bond between the HMG and the CoA moiety (Fig. 1). The aim of this study was to investigate the possible existence and magnitude of isotope discrimination by HMGR in order to provide better understanding of the factors that control the ¹³C/¹²C ratios of sterols, steroids, and other products of the mevalonate pathway.
2 Experimental

2.1 Vector design

A plasmid containing the gene for human HMGR was purchased from the American Type Culture Collection. The catalytic subunit (residues 420-888 [8]) was amplified by PCR introducing an AflIII and BamHI restriction site at the ends (restriction enzymes from New England Biolabs). The fragment was inserted into the pNHis vector [9] between the NcoI and BamHI restriction site using standard ligation procedures with T4 DNA ligase (Roche). The pNHis vector introduces an N-terminal polyhistidine tag which permits affinity purification. The ligation mixture was transformed into Escherichia coli DH5α.

Vectors containing the insert were identified by agarose gel electrophoresis of minipreparations of plasmid DNA from single colonies. A suitable vector was amplified in DH5α cells (Qiagen plasmid midi kit). To avoid fragmentary expression starting from an internal translation initiation site [10] M485 was mutated to I485 by side directed mutagenesis (reagents from NEB). According to Istvan et al. the enzymatic activity of the M485I mutant protein is unchanged compared with the wild-type protein [8].

2.2 Protein expression and purification

For expression of the fusion protein, the vector was transformed into Escherichia coli BL(DE3). Cells were grown in LB medium [11] at 37°C until they reached an OD_{600} of 0.8, then induced with 1 mM IPTG and grown at 25°C for 4 h. Purification was carried out using a Ni-NTA Agarose affinity column (Qiagen), and according to a vendor provided protocol. Protein con-
centrations were determined by the method of Bradford [12]. The two main fractions were combined, concentrated by centrifugal filtration (Millipore amicon ultra 30.000) and rebuffered in potassium phosphate buffer [13] (0.2 M KCl, 0.16 M potassium phosphate, 0.004 M EDTA, and 0.01 M DTT, pH 6.8). Enzyme purity was checked by SDS-PAGE. For storage, the solution was spiked with 10% (v/v) glycerol and aliquots of 200µL were kept at −20°C.

2.3 Enzymatic reaction

The reaction system consisted of 160 mM potassium phosphate (pH 6.8), 200 mM KCl, 420 myM NADPH (Sigma), and 311 myM (R,S)-HMG-CoA (Fluka) in a final volume of 800µL. The reaction was initiated by adding the enzyme to the complete assay mixture in a volume that was sufficient to complete the conversion within approximately 14 minutes. The HMG-CoA-dependent oxidation of NADPH was monitored at 340 nm in a spectrophotometer (Pharmacia Biotech Ultrospec 2000) equipped with a cell holder adjusted at 37°C. Up to three samples of 250µL per run were taken and transferred into ice cooled glass vials. The vials had been equipped with a solution of pravastatin (Sigma) to inhibit further reaction. For samples taken at fractions of reaction smaller than 0.15, volumes of 500µL were pipetted.

2.4 Sample preparation

For lactonization of mevalonic acid, the samples volume was doubled by addition of 0.1 M HCl and the reaction was left unattended at room temperature for about 1 hour. Solid-phase extraction was conducted as described by Saini et al. [14], with slight modifications. Briefly, the samples were individually
transferred to a solid-phase extraction cartridge (Separtis Isolute ENV+ 100 mg/3 ml) that had been washed and preconditioned with 3 mL methanol followed by 1 mL water. The sample was allowed to enter the column bed by gravity flow and the column was washed with 500 μL HCl followed by 500 μL water. The cartridges were dried under gentle vacuum suction. The column bed was conditioned with 500 μL methanol, and another volume of 500 μL eluted the analytes. The methanol extract solution was dried in a vacuum dessicator over phosphorus pentoxide. For GC-C-IRMS analysis analytes were taken up in individually determined volumes of tert-butyl methyl ether, in order to minimize concentration varieties.

2.5 Stable isotope analysis

Stable carbon isotope analysis of MVL was performed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

The hardware encompassed an Agilent GC 6890 (Agilent Technologies) coupled to a Thermo Delta-plus XP gas isotope ratio mass spectrometer. The system was largely modified as described by Flenker et al. [15].

Briefly, the solvent removal, which is essential to GC-C-IRMS, is performed before passage of the GC column. A Gerstel cooled injection system KAS-4 (Gerstel) supported by an auxiliary vacuum is employed for this purpose.

Solvent removal was achieved at a temperature of 5°C within an interval of one minute. The vent flow was adjusted to 50 mL/min and the auxiliary vacuum was set to -0.1 bar. The sample transfer to the GC column was performed at 200°C within another minute. The material was recondensed on the column.
at 35°C. Subsequently the injection port was purged at a temperature 300°C where the gas flow was set to 100 mL/min. The temperature rates between the different steps amounted to 10 K/s.

The GC column was a Macherey & Nagel Optima-1701. The dimensions were 30 m length, 320 µm inner diameter, and 0.5 µm film thickness. The carrier gas flow was set to 1.8 mL/min, where constant flow mode was used.

The initial GC temperature (35°C) was maintained for 2 min. The temperature then was raised to 135°C at 40 K/min. Subsequently, the temperature was changed to 250°C at a rate of 10 K/min.

Typically, two reference gas pulses were employed to calculate the 13C/12C ratio of MVL. For this purpose, one uncontaminated pulse before and one after the MVL signal were selected. Figure 2 shows a typical analysis and illustrates the procedure. The reference gas was calibrated versus a n-alkane mixture kindly provided by the University of Indiana [16].

2.6 Data evaluation

The spectrophotometric absorbance was plotted against time. During sampling the cover of the spectrophotometer had to be opened. Data measured within these intervals were removed from the plot. A cubic smoothing spline [17] was fitted to the remainder of the data. It was assumed that the observed decrease of NADPH concentration depended on two different factors, the consumption of NADPH during the enzyme reaction and the enzyme-independent decay of NADPH. The latter depends on pH and temperature [18].

In order to isolate the enzymatic effect, the curve was corrected by addition
of the absolute difference of two decay lines. The latter ones individually were
calculated according to the NADPH concentration before and after complete
conversion of (S)-HMG-CoA, respectively. The fraction of reaction then was
calculated as the ratio of the difference between initial absorbance and ab-
sorbance at the time of sampling divided by the total difference of absorbances
(Fig. 3).

The fractionation factor (α) and the isotopic ratio of the product molecule
after complete conversion (R∞) were estimated by nonlinear least squares
curve fitting. The software was R in its latest version where the nls-library
was employed [19].

The model formula was derived from the rate laws of the stable carbon isotopes
(see appendix):

\[ R_t = \frac{1 - \exp\left(\frac{\log(1 - f)}{\alpha}\right)}{f} \cdot R_\infty \]  

(1)

R_t is the isotopic ratio of the sample at time t and f is the corresponding
fraction of reaction.

The KIE of the enzyme reaction is then given by

\[ \text{KIE} = (\alpha - 1) = \left(\frac{k^{12}}{k^{13}}\right) - 1, \]  

(2)

where \(k^{12}\) and \(k^{13}\) indicate the rate constants for \(^{12}\)C and \(^{13}\)C, respectively.
3 Results and Discussion

Figure 2 shows a typical GC-C-IRMS analysis of MVL following our protocol for enzymatic production, sample preparation, and $^{13}$C/$^{12}$C analysis.

In this case the reference gas pulses R3 and R5 were employed to calculate the $^{13}$C/$^{12}$C of MVL. The contamination of R4 appeared irregularly and probably results from the solid phase extraction. The MVL signal itself is clearly resolved and certainly yields valid results. Because the HMGR reaction was stopped after varying intervals, the resulting MVL concentrations were rather different. The required amounts of solvent however were estimated in advance and thus approximately identical peak heights could be obtained. In fact these varied by less than a factor of 3 which falls well within the linear dynamic range of IRMS analyses.

Figure 4 shows the observed relation between $f$ and $R_t$. The fitted curve according to equation (1) is plotted. Analysis of the residuals suggested presence of some outliers. In fact two outliers were confirmed by Huber statistics [20]. The curve was refitted after removal of these points. The corresponding estimates for $\alpha$, $R_\infty$, and the calculated KIE are listed in table 1.

The standard deviation of the residuals is close to the known repeatability precision of GC-C-IRMS analyses. Moreover, the residuals are independent from $f$ and exhibit random scatter. It can therefore be assumed that the relation between $R$ and $f$ is described adequately.

Both estimates for $\alpha$ and $R_\infty$ and their corresponding confidence intervals could be confirmed by application of bootstrap methods [21].
KIEs are commonly determined [22–24] by the method of internal competition [1]. The calculation employs the equation proposed by Tong [25] or its simplified version by O’Leary [26], both describing $\alpha$ in terms of $f$, $R_t$, and $R_\infty$. This method requires several measurements of the isotopic composition $R_t$ of the starting material or the reaction product over the range from as little as 1% reaction to as much as 50% reaction. Secondary the value of $R_\infty$, which is the isotopic composition of the product at total conversion, must be known.

Because of the steep dependence of the product isotopic composition at the end of the reaction (see figure 1) $R_\infty$ is afflicted with large experimental errors and can be regarded as the major source of bias. However, the curve fitting method used in this study does not require any exact value for $R_\infty$. In fact $R_\infty$ and $\alpha$ are estimates obtained from the fitting process.

The model equation, which was used to calculate the KIE, is based upon the assumption of a pseudo first-order irreversible enzyme reaction. Human HMGR utilizes two equivalents of NADPH as a cosubstrate. In order to meet the first-order demand limitation by a substrate other than HMG-CoA was avoided by excess NADPH.

The calculated KIE has to be treated as the average isotope effect on all carbon atoms contained in the product molecule. Assuming that the isotope effect

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>1.0031</td>
<td>±4 · 10^{-4}</td>
<td>±8 · 10^{-4}</td>
</tr>
<tr>
<td>$R_\infty$</td>
<td>1.0917 · 10^{-2}</td>
<td>±3 · 10^{-6}</td>
<td>±5 · 10^{-6}</td>
</tr>
<tr>
<td>$KIE$</td>
<td>3.1‰</td>
<td>±0.4‰</td>
<td>±0.8‰</td>
</tr>
</tbody>
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Table 1
Estimates for $\alpha$ and $R_\infty$. SE: standard error, CI: confidence interval (95%).
would be largely restricted to C-1 and only normal isotope effects contribute
to the observed value, the maximum expected depletion of $^{13}$C at C-1 can
be assessed at least roughly. Thereto the average isotope effect is multiplied
with the number of carbon atoms present in the product molecule, in this
case six for mevalonolactone. The resulting value of 18.6‰ is not unusual in
enzymatic reactions where carbon bonds are cleaved. For example, bacterial
decarboxylases were reported to exhibit KIEs between 14.7 and 18.2‰ at
C-1 [22,26].

However, Melzer & Schmidt [27] investigated the pyruvate dehydrogenase re-
action from two different organisms and calculated position specific isotope
effects. In fact, $^{13}$C depletions from 9 to 24‰ and from 21 to 25‰ were ob-
erved for C-1 and C-2, respectively. C-3 still revealed depletions around 3‰.

From the above it is certainly inadequate to attribute the entire isotope effect
of HMGR to C-1. The effect would rather show some kind of distribution be-
tween the three atoms C-1, C-2, and C-3 (Fig. 1). Nonetheless, a calculated
propagation of $^{13}$C depletion from mevalonate to cholesterol would remain
unaffected. Cholesterol incorporates all three of the carbon atoms concerned
from six molecules of mevalonate. The depletion would therefore propagate
with $18.6 \times 6/27 = 4.1‰$ to the C$_{27}$-body of cholesterol (Fig. 5). Likewise,
pregnanes incorporate five groups of C-1 to C-3 and would show a $^{13}$C deple-
tion of about $18.6 \times 5/21 = 4.4‰$.

In contrast, androstanes incorporate C-1 and C-2 from mevalonate at equal
numbers, but lack one atom C-3 (Fig. 5). The expected $^{13}$C depletion of this
compound depends on the actual effect attributed to C-3. Considering it to
fall between one third and zero of the overall KIE, androstanes would exhibit
depletions between $(18.6 \times 5 - 18.6/3)/19 = 4.6‰$ and $18.6 \times 5/19 = 4.9‰$,
respectively. All of these numbers represent estimates which rely on sole limitation of steroid biosynthesis by HMGR.

The results are of practical relevance for doping control, where stable isotope ratios are used to identify the primary sources of steroids. The method compares the $^{13}\text{C}/^{12}\text{C}$ ratios of androgens or corresponding metabolites to those of steroids from independent pathways. For example, testosterone (T, C$_{19}$) may be compared to pregnanediol (C$_{21}$). The latter thus serves as “endogenous reference compound”. Synthetic androgenic steroids betray their origin in that their $^{13}\text{C}/^{12}\text{C}$ values significantly differ from those of the endogenous reference compounds. Up to now, with very few exceptions only $^{13}\text{C}$-depletions have been observed for synthetic steroids.

Consequently, during the application of synthetic T, the $^{13}\text{C}/^{12}\text{C}$-ratios of T itself and its metabolites will be reduced compared to the purely biosynthetic pregnanes. Depending on the considered target and reference compounds, a difference of at least 3‰ on the $\delta$-scale$^1$ must be exceeded in order to tell the presence of synthetic steroids [31].

The described doping control methodology presumes that physiological factors do not significantly increase the corresponding differences in stable isotope composition. As demonstrated above, full limitation by HMGR could result in a maximum difference in $^{13}\text{C}$ depletion of 4.9 − 4.4 = 0.5‰ when comparing C$_{19}$- and C$_{21}$-steroid hormones. Therefore $^{13}\text{C}$ discrimination by HMGR would only be able to slightly quench the $^{13}\text{C}$ enrichment attributed to doping. It can

$^1$ The $\delta^{13}\text{C}$ value is defined as:

$$\delta^{13}\text{C}(‰) = \left(\frac{[^{13}\text{C}]/[^{12}\text{C}]_{\text{sample}}}{[^{13}\text{C}]/[^{12}\text{C}]_{\text{standard}}} - 1\right) \cdot 1000$$

The standard is carbon dioxide from PeeDee Belemnite isotopic standard limestone [29].
be stated that the influence of the HMGR isotope effect will not significantly exceed the analytical error generally present in GC-C-IRMS. It is unlikely that corresponding results will be corrupted by the described phenomena. Nonetheless, it should be kept in mind that different steroids, although originating from an identical pathway do not necessarily exhibit identical stable isotope signatures. Since the atoms of mevalonate will have a non-statistical distribution of $^{13}$C, the measured average depletion depends on the atoms incorporated into or removed from the analysed molecule.

Apart from practical issues, our results allow to draw interesting conclusions concerning the nature of steroid biosynthesis. Crude lipids were found to be depleted in $^{13}$C by 7‰ relative to biomass [4,28]. The rate limitation of acetate formation catalyzed by pyruvate dehydrogenase is likely to be responsible for this phenomenon [27]. However, amongst lipids, free fatty acids exhibit considerable $^{13}$C/$^{12}$C-depletions of around 8‰ whereas steroids are only sightly depleted by ca. 1‰ compared to biomass [6]. The lack of $^{13}$C-depletion rules out rate limitation of steroid formation by the decarboxylation of pyruvate.

It is usually assumed that HMGR features the rate limiting step in sterole synthesis. In the present study of the HMGR reaction, a depletion propagating with 4–5‰ to sterols has been calculated. Considering that sterols appear much less depleted than can be predicted from this result it may be concluded that under physiological conditions, most of the HMG-CoA emerging in the relevant compartment is converted to mevalonate. This would result in disappearance of significant HMGR induced isotope fractionation for the most part.

In general, the influences of in vivo isotope effects are modulated by various factors such as the division of carbon fluxes to alternative metabolic pathways
or compartmentalization. In order to interpret patterns of isotopic depletion
the structure and characteristics of the related network of reactions has to be
considered [3]. Thus the results presented contribute to the interpretation of
isotopic patterns in the mevalonate pathway.
We presume a system of pseudo first-order irreversible reactions of the form \( A \rightarrow B \) with the rate constant \( k \). For molecules with different isotopic composition at positions that influence the reaction rate we assume for the lighter carbon isotope \(^{12}\text{C}\) and for the heavier carbon isotope \(^{13}\text{C}\), respectively:

\[
A' \xrightarrow{k'} B' \quad \text{and} \quad A'' \xrightarrow{k''} B''
\]

The mass balance demands \([A] = [A'] + [A'']\) and \([B] = [B'] + [B'']\). That means all further relations can be applied to the respective isotope by substitution of \( A, B \) and \( k \). The concentration of the product \([B]\) increases as the substrate concentration decreases. At time \( t \) the product concentration is given by:

\[
[B]_t = [A_0] - [A_t]
\]

The reaction rate of the pseudo first-order reaction depends on the concentration of the substrate:

\[
d[A_1]/dt = -k \cdot [A_1]
\]

\[
[A_1]/[A_0] = \exp(-k \cdot t)
\]

The total reaction rate equals the sum of the two rate terms:

\[
d[A_t]/dt = d[A'_1]/dt + d[A''_1]/dt
\]

\[
[A_t] \cdot k = [A'_1] \cdot k' + [A''_1] \cdot k''
\]

At any time in the course of the reaction the \(^{12}\text{C}\) abundance is approximately a factor of \(10^2\) higher than the \(^{13}\text{C}\) abundance and changes in this relation
occur beyond the $10^2$-scale. Therefore it was approximated that $[A_t'''] \cdot k'''$ was small compared to $[A_t'] \cdot k'$ and could be excluded from further considerations. This approximation yields the following relation:

$$k = k'$$  \hspace{1cm} (5)

The fraction of reaction is given by the amount of B formed during the course of reaction. It was normalized by the final concentration of the product $[B_\infty]$, which equates to the starting concentration of the substrate $[A_0]$.

$$f = [B_t]/[A_0]$$  \hspace{1cm} (6)

$$f = 1 - [A_t]/[A_0] \quad \text{; with equation (3)}$$

$$f = 1 - exp(-k \cdot t) \quad \text{; with equation (4)}$$  \hspace{1cm} (7)

From this an expression for time $t$ is derived:

$$t = -log(1 - f)/k$$

$$t = -log(1 - f)/k' \quad \text{; with approximation (5)}$$  \hspace{1cm} (8)

By combining equation (6), (7), and (8) the product concentration is given by:

$$[B_t] = (1 - exp(-k \cdot (-log(1 - f)/k')))) \cdot [A_0]$$  \hspace{1cm} (9)

The isotopic ratio of the product is defined as the quotient of the concentrations of the heavier and the lighter isotope:

$$R_t = [B_t''']/[B_t']$$  \hspace{1cm} (10)
Yet another approximation took into account that in practice the calculation of the fraction of reaction $f$ was afflicted with errors that would exceed deviations between the $f$s:

$$f = f' = f''$$  \hspace{1cm} (11)

By adapting equation (9) to the different isotopes and insertion into equation (10) we obtain:

$$R_t = \frac{1 - \exp(-k'' \cdot (-\log(1 - f)/k')) \cdot [A_0']}{1 - \exp(-k' \cdot (-\log(1 - f)/k''))} \cdot \frac{[A_0']}{[A_0]}$$  \hspace{1cm} (12)

The quotient $[A_0']/[A_0]$ corresponds to the isotopic ratio of the product after complete reaction, referred to as $R_\infty$. The rate constants can be substituted using the definition $\alpha = (k'/k'')$. Thereby $R_t$ can be expressed as function of $f$ with the two parameters $\alpha$ and $R_\infty$:

$$R_t = \frac{1 - \exp(\log(1 - f)/\alpha)}{f} \cdot R_\infty$$  \hspace{1cm} (1)
References


Figure legends

**Figure 1:** Enzyme reaction of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). Isotope effects are expected at C-1, C-2, and C-3, the group of atoms adjoining the cleaved C-S bond.

**Figure 2:** GC-C-IRMS analysis of MVL isolated from the HMGR reaction mixture. R1–R5: reference gas pulse; MVL: mevalonolactone, UNK: unknown contaminant. R3 and R5 were used to calculate the $^{13}\text{C}/^{12}\text{C}$ of MVL in this run.

**Figure 3:** Determination of the fraction of reaction $f$ from spectrophotometric data. Decay lines DL0 and DL1: estimated enzyme independent change of absorbance for $f=0$ and $f=1$ respectively; Curve P: cubic smoothing spline fitted to absorbance data; $t_1$ to $t_2$: interval evaluated for linear regression and calculation of DL0; $t_3$: enzyme addition; $\Delta A(t_3)$: difference of absorbance due to complete conversion of (S)-HMG-CoA; AP(x): absorbance of sample x; Af0: assumed absorbance for $f=0$; Af1: assumed absorbance for $f=1$; Fraction of reaction of sample x: $f=(\text{AP} - \text{Af0})/(\text{Af1} - \text{Af0})$.

**Figure 4:** Observed relationship between $R = ^{13}\text{C}/^{12}\text{C}$ of MVL and the fraction of reaction $f$. Two outliers plotted as crossed points were removed from the dataset. The dashed line marks the value of $R_\infty$.

**Figure 5:** Cholesterol incorporates carbon atoms C-1 to C-3 from six mevalonate molecules. The denoted positions 1 to 3 refer to the mevalonate molecule.
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