**Effect of substrate features and mutagenesis of active site tyrosine residues on the reaction course catalysed by Trypanosoma brucei sterol C-24-methyltransferase**

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**INTRODUCTION**

Much of the structural diversity found in the sterol biosynthetic pathway is introduced by olefin C-methylation reactions of a few common Δ24 substrates catalysed by 24-SMT (sterol C-24-methyltransferase) to form 24-alkyl sterols [1]. In the biosynthesis of these ancient biomolecules [2–4], which function primarily as architectural components of membranes [5–7], the size and configuration of the 24-α-keto group has phylogenetic significance; the β-methyl group is typically synthesized in early divergent organisms represented by protozoa and fungi, the α-ethyl group synthesized in vascular plants and the C11-β:β extended side chain in chrysophyte algae and marine invertebrates (Figure 1) [8–10]. These 24-SMT enzymes of tetrameric organization utilize different substrate (Supplementary Figure S1 at http://www.BiochemJ.org/bj/439/bj4390413add.htm) and product specificities, but in their primary substrate share similar sequence identity across kingdoms (49–77%) and three conserved regions coinciding with substrate-binding segments for sterol acceptor molecules [11]. This feature, the cationic nature of C-methylation-deprotonation reactions, and mutagenesis studies of fungal and plant 24-SMTs targeted at contact amino acids have shown that specifically spaced tyrosine residues in the active site contribute electron-rich aromatic amino acids to catalysis, where they direct the conformation of the flexible sterol side chain and stabilize positively charged transition states during the reaction sequence.

In spite of a complete isoprenoid-sterol biosynthetic pathway including the 24-SMT gene present in all sequenced Trypanosomatidae genomes [12–14], there is a unique 24-alkyl sterol composition in Trypanosoma brucei, the causative agent for African sleeping sickness. According to co-metabolite and substrate-specificity studies of the TbSMT (24-SMT from T. brucei) and sterol 14-demethylase enzymes, lanosterol is converted into 31-norlanosterol then ergosterol, and its structural isomers ergosta-5,7,25(27)-trienol, ergosta-5,7,24(25)-trienol and 24 dimethyl ergosta-5,7,25(27)-trienol. We made use of our prior research and molecular modelling of 24-SMT to identify contact amino acids that might affect catalysis. Conserved tyrosine residues at positions 66, 177 and 208 in TbSMT were replaced with phenylalanine residues. The substitutions generated variable loss of activity during the course of the first C-1-transfer reaction, which differs from the corresponding Erg6p mutants that afforded a gain in C-2-transfer activity. The results show that differences exist among 24-SMTs in control of C-1- and C-2-transfer activities by interactions of intermediate and aromatic residues in the activated complex and provide an opportunity for rational drug design of a parasite enzyme not synthesized by the human host.

Key words: homology modelling, kinetic isotope effect, mutagenesis, sterol C-24-methyltransferase (24-SMT), Trypanosoma brucei, zymosterol.

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**Abbreviations used:** AdoMet, S-adenosyl-L-methionine; a.m.u., atomic mass unit; CMA, mycolic acid cyclopropane synthase; EI-MS, electrospray ionization-MS; FR, fold recognition; KIE, kinetic isotope effects; RFM, Rossmann-fold MFAse; RTTc, retention time relative to retention time of cholesterol; 24-SMT, sterol C-24-methyltransferase; TbSMT, 24-SMT from Trypanosoma brucei.

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sterols and efforts to develop such agents are currently underway in several laboratories [15,18–22].

The present paper describes new details of the substrate scope for multiple product TbSMT, and through homology modelling and mutagenesis experiments key tyrosine residues were identified to be involved in catalytic competence. These studies were facilitated by assay of 24-2H- and 27-13C-labelled sterols, performed previously with fungal and plant 24-SMTs [23], which allowed an explanation for timing of the C-methylation steps and regiospecificity associated with the TbSMT-generated products. The results support the hypothesis that TbSMT represents a primitive type of 24-SMT enzyme which evolved a unique active-site motif from those described previously. Moreover, the present paper is the first report to show the relevance of substrate selectivity directing the sequence of enzymatic activities in ergosterol biosynthesis.

EXPERIMENTAL

Substrates

Zymosterol, [24-2H]zymosterol (98% atom enrichment) and [27-13C]zymosterol (98% atom enrichment) were prepared as described previously [24]. 24-Methyldesmosterol [ergosta-5,24(25)-dienol] was prepared by acid-induced isomerization of 24(28)-methylenecholesterol obtained from corn pollen [25]. Cholesta-5,7,24-tirenol and cholesta-5,7,22,24-tetraenol were isolated from the yeast mutant Erg6 [26]. [methyl-3H]AdoMet (AdoMet is S-adenosyl-L-methionine; 99% atom enrichment) was from MSD Isotopes, [methyl-3H]AdoMet (diluted to 10 μCi/μmol) was purchased from New England Nuclear and AdoMet as the chloride salt was from Sigma. Sterol substrate purity was approximately >95% by GC analysis. All other reagents and chemicals were purchased from Sigma or Fisher unless otherwise noted.

Table 1 DNA sequence of the synthetic oligonucleotides used for the site-directed mutagenesis of the TbSMT gene

<table>
<thead>
<tr>
<th>Strain</th>
<th>Forward oligonucleotide sequence (5′−3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y166F</td>
<td>gttacagatttcttcgaatacggttgg</td>
</tr>
<tr>
<td>Y177F</td>
<td>ttgcagcagcgcatgcatgcatgaggcg</td>
</tr>
<tr>
<td>Y208F</td>
<td>atcgaacctggtgctcactttaagctg</td>
</tr>
</tbody>
</table>

Site-directed mutagenesis of TbSMT

Site-specific mutations at various positions along the pCW-ERG6 template were carried out using the QuikChange® site-directed mutagenesis kit (Stratagene). The replaced mutant codons are listed in Table 1. Putative positive clones were picked, the plasmids isolated using the Promega SV wizard miniprep kit and the mutation confirmed by sequencing using the appropriate primer. Vectors carrying the mutated gene for 24-SMT were transformed into the expression host Escherichia coli BL21 (DE3) (Novagen) as described previously [25].

TbSMT assay and product analysis

E. coli (BL21) harbouring recombinant wild-type or mutant TbSMTs were harvested by centrifugation (13 300 g for 10 min at 4°C) and the cell pellet lysed by French-press disruption as described previously [25]. The resulting crude TbSMT in the soluble fraction (300 μl with 3 mg of total protein) was assayed in the presence of sterol substrate either at saturation (100 μM) or varied over the concentration range (5–150 μM) emulsified with Tween 80 (final concentration 1.2 g/l). Incubations of a total volume of 600 μl were initiated with AdoMet (100 μM of 100 μM; in kinetic studies AdoMet was diluted with 0.6 μCi of
Table 2 Observed KIE for deprotonation and products catalysed by TbSMT

Rates are the averages of duplicate assays, using the same lystate preparation for all three substrates, performed under standard incubation conditions for 1 h. Results were fitted non-linearly to the Michaelis–Menten equation using the enzyme kinetic module from SPSS; accordingly, the mean standard error of the \( V_{\text{max}} \) determinations was ±3 pmol·min\(^{-1}\)·mg\(^{-1}\) and \( r^2 = 0.95.\) For enzyme-generated product analysis identical assay conditions were used except that the assay continued for 16 h. Values in parentheses are the catalytic competence normalized to 100% in reference to zymosterol or wild-type.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Steady-state kinetic parameters</th>
<th>Relative proportion of TbSMT generated products (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} ) (pmol·min(^{-1})·mg(^{-1}))</td>
<td>( K_m ) (µM)</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>258</td>
<td>55</td>
</tr>
<tr>
<td>[27-(^{13})C]zymosterol</td>
<td>258</td>
<td>55</td>
</tr>
<tr>
<td>[24-(^{3})H]zymosterol</td>
<td>215</td>
<td>60</td>
</tr>
</tbody>
</table>

\([\text{methyl-}^{2}\text{H}]\text{AdoMet}\) and continued aerobically at 35°C with gentle shaking for 60 min. During this period the progression of the reaction was linear. The reaction was stopped by the addition of 600 µl of 5% (w/v) KOH-methanol. Sterols were extracted three times with hexane and the combined extracts concentrated to dryness. For every substrate, the kinetic parameters of the reaction (\( K_{\text{m,app}} \) and \( V_{\text{max,app}} \)) determined under initial velocity conditions (Supplementary Figure S2 at http://www.BiochemJ.org/bj/439/bj4390413add.htm) were calculated by fitting the liquid scintillation data to the Michaelis–Menten equation using the enzyme kinetic module from SPSS. The error in work-up of duplicate samples was generally 5–10%. Controls, containing radioactive AdoMet and no sterol, were used to background-subtract methylation activity due to endogenous AdoMet-dependent enzymes in the lystate; generally the accumulation of radioactivity in these treatments was low, 500–1000 d.p.m. For use of isotope effects to determine enzyme mechanisms, the same batch of lystate protein was employed in incubations of \( ^{2}\text{H}-\) and \( ^{13}\text{C}\)-labelled zymosterols and zymosterol.

Sterol compounds, referenced to the retention time of cholesterol in capillary GC at 13.8 min and HPLC at 22 or 37 min (analytical compared with semi-preparative C\(_{18}\) column), were quantified by integration of the detector signal from 10–30 min. Products were routinely identified by their retention times in GC and electron-impact spectrum with those of reference specimens. For selected products, unambiguous identification was established after HPLC (Phenomenex C\(_{18}\) column linked to a diode array detector which provided UV spectra) fractionation followed by GC-MS analysis (HP LS 6500 gas chromatograph interfaced to a 5973 mass spectrometer) and NMR [spectra measured on deuteriochloroform solutions using a Varian Unity Inova 500 MHz spectrometer operating at 500 MHz for protons and 125 MHz for \(^{13}\text{C}\) nuclei; chemical shifts were referenced to CHCl\(_3\) at \( \delta \) 77.00 and reported as \( \delta \) (p.p.m.)] identification of the biomethyl sterol as described previously [24–27].

Protein sequence analysis and structure prediction

Multiple amino-acid sequences of diverse 24-SMTs were aligned with ClustalW2 at the European Bioinformatics Institute with default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Protein structure prediction was carried out via the GeneSilico MetaServer [28], which is a gateway for a variety of bioinformatics algorithms for prediction of secondary structure, intrinsic disorder, per-residue solvent accessibility and protein FR (fold recognition), i.e. matching the target sequence to known protein structures (potential templates). The potentially best template has been identified according to the consensus between different FR methods. Top-scoring pairwise alignments between the TbSMT sequence and the structure of the best template were used as a starting point for comparative modelling. We used the ‘FRankensteins’s Monster’ approach which comprises cycles of local realignments in uncertain regions, building of alternative models and their evaluation, realignment in poorly scored regions and merging of the best-scoring fragments [29,30]. Previously, we used this approach for successful building of models for different methyltransferases that were later confirmed by crystallographic analyses [31,32]. Diverged regions of TbSMT, for which the FR analysis failed to identify consensus alignments, and whose predicted secondary structure differed significantly from that of the template, have been remodelled using de novo folding methods of ROSETTA [33] and REFINER [34]. Mapping of sequence on to the model was done for the multiple sequence alignment of the 24-SMT family with the ConSurf server [35].

Bioinformatics analysis identified members of the RFM (Rossmann-fold Mtase) superfamily [36] as the best modelling templates, with the crystal structure of CMA (mycolic acid cyclopropane synthase), CmaA2 from Mycobacterium tuberculosis (PDB code 1KPI) [37], reported with the highest scores by most methods. We have built a preliminary model of TbSMT using a comparative (homology-based) method, based on alignments obtained by the GeneSilico Metaserver, docked the ligands (AdoMet and sterol molecule) based on superimposition on to the co-substrates present in the template structure, and optimized the conformation of the variable regions using de novo folding methods. The final model of TbSMT was evaluated as a ‘very good model’ using the PROQ method [38] with a score of 0.413, and MetaMQAP predicted that its root mean square deviation to the wild-type structure is approximately 3.9 Å (1 Å = 0.1 nm) [39].

RESULTS

General features of the TbSMT-catalysed reaction

The structures and distribution of the TbSMT-catalysed reaction of zymosterol have been described for the recombinant sterol gene introduced into E. coli HMS-174 cells [16]. In brief, GC-MS analysis of the enzyme-generated products from a soluble TbSMT showed the presence of four biomethylated sterols identified as ergosta-8,25(27)-dienol, ergosta-8,24(28)-dienol, ergosta-8,24(25)-dienol and 24 dimethyl ergosta-8,25(27)-dienol in a ratio of 7:3; the products distributed in a ratio of approximately 5:1:2:2 respectively. In the present study, the TbSMT expressed in E. coli BL21 cells generated a sterol composition much like that reported previously (Table 2). However, using a lystate rather than soluble preparation, the rate of the reaction for zymosterol catalysis was found to be somewhat lower than determined before at a \( V_{\text{max}} \) value of 258 pmol·min\(^{-1}\)·mg\(^{-1}\).
compared with a \( V_{\text{max}} \) value of 901 pmol·min\(^{-1}·mg \), whereas binding in the Michaelis complex was similar to that established previously with a \( K_m \) value of 55 ± 5 \( \mu \)M compared with 47 ± 5 \( \mu \)M (Table 2).

The previous \(^1\)H-NMR evaluation of the \( Tb\)SMT-generated ergosta-8,25(27)-dienol revealed the 24-methyl group to be \( \beta \)-oriented [16]. In the present study, we evaluated the deprotonation steps in the C-24-methylation mechanism directly, by a comparison of methyl→methylene elimination in formation of the \( \Delta^{24(28)} \) - and \( \Delta^{25(27)} \) -olefins by \( Tb\)SMT using [27-\(^{13}\)C]zymosterol and zymosterol. As expected, the rates of catalysis for both substrates and product distributions were similar; the molecular ion cluster of peaks in the mass spectra of the four 

\[ \text{substrate utilization of } \text{C-1,2-transfer reactions catalyst by } \text{TbSMT, three substrates with } \Delta^\text{-bonds were tested with the recombinant enzyme, ergosta-5,24(25)-dienol (24-methyl desmosterol), cholesta-5,7,24-trienol and cholesta-5,7,22,24-tetraenol. The sterols were individually bound by the } \text{24-SMT such that each one of them converted into a biomethyl product of similar binding constants of a } K_m \text{ value if } 25 ± 3 \( \mu \)M, but they differed in their rates of conversion with zymosterol (\( V_{\text{max}} = 258 \text{ pmol/min} \)) catalysed faster than ergosta-5,7,22-trienol (\( V_{\text{max}} = 155 \text{ pmol/min} \)) or cholesta-5,7,22,24-tetraenol (\( V_{\text{max}} = 33 \text{ pmol/min} \)) and ergosta-5,24-dienol (\( V_{\text{max}} = 74 \text{ pmol/min} \)). GC-MS analysis of the reaction products showed that: (i) ergosta-5,24-dienol converted into a single product at a RRTc value of 1.27 of a M\(^+\) value of 380 a.m.u. and a minor compound identified previously from yeast as ergosta-5,24-dienol [RRTc value of 1.15 and a \( M^+ \) value of 394 a.m.u.] and other diagnostic ions at 380 a.m.u. (100),
Sterol C-24-methylation reaction

Information from homology modelling

The three-dimensional structure of any orthologue of TbSMT is at present unknown, yet over 100 structures of AdoMet-dependent methyl transferase have been reported that can be used as reference material in homology modelling of a 24-SMT that binds sterol along with AdoMet. To ascertain mechanistic inferences on the involvement of a cationic intermediate in catalysis by mutants prepared in the present study, we undertook modelling and docking experiments of the TbSMT to show putative interaction between protein and co-substrates sterol and AdoMet in the active site. The current modelling studies take into consideration our investigations that involved photoaffinity and chemical labelling and site-directed mutagenesis experiments in the Erg6p [27,43–45]. The combination of these efforts reveal four substrate-binding segments for AdoMet and sterol referred to as Regions I–IV [45], and that tyrosine residues at positions 81, 192 and 223 (equivalent to Tyr86, Tyr177 and Tyr223 in TbSMT) represent contact amino acids for sterol-binding sites (Figure 6 and Supplementary Figure S6 at http://www.BiochemJ.org/bj/439/bj4390413add.htm).

Using a refined and updated set of sequence and structure information, allowed us to construct a pseudo-atomic model of a 24-SMT which is in general agreement with prior mutagenesis of Erg6p that revealed four substrate-binding segments associated with the active site (Figure 7). The new bioinformatics analysis predicts that a protomer of TbSMT forms a relatively compact structure, formed by the highly conserved RFM domain [36,46] surrounded by variable elements. In the ligand-bound conformation based on the related CMA structure, the enzyme is in the closed conformation, with the AdoMet-binding pocket covered by the N- extension and the sterol-binding pocket covered by insertions in the catalytic domain. The key residues involved in binding of AdoMet to TbSMT (in parentheses are the Erg6p residues) appear at His75 (His90) and Asp110 (Asp125), corresponding to substrate domains for Regions I and II, which co-ordinate the methionine moiety, and Asp162 (Asp177) which co-ordinates the adenosine moiety. Prediction of sterol-binding residues is more difficult, as they are specific to the 24-SMT family and absent in other members of the RFM superfamily. Furthermore, the conformation of the sterol side chain to ligand binding is enigmatic. With these considerations in mind, mapping of residues that are evolutionarily conserved just in the 24-SMT family on to the structure of our model reveals a putative substrate-binding pocket.

It is worth mentioning that our model refers to a structure of an isolated SMT protomer, although it is known based on gel-permeation chromatography that the functionally active form of the protein is a tetramer [16,40]. Without experimental structural information (such as the protein shape or sites of contact between different SMT protomers in the tetramer) it is currently impossible to reliably predict the quaternary structure. It is probable that some protein segments, especially the regions outside the conserved RFM core, may assume a different conformation in the tetramer rather than in our model. However, we believe that tetramerization is unlikely to affect the protein core that harbours the active site, and hence we are relatively confident in our prediction of key functional residues.

Effect of tyrosine to phenylalanine mutations on TbSMT activity

Based on the interactions observed in the model and earlier studies targeted at Erg6p tyrosine residues at positions 81, 192 and 223, which upon mutation to phenylalanine recognizes Δ24(28) substrates and converts them into multiple 24-ethyl(idene) products [45], the TbSMT residues at Tyr86, Tyr177 and Tyr223 were
replaced with phenylalanine. These amino acid replacements, to conserve size and shape but eliminate the potentially reactive function, did not act like the corresponding Erg6 mutant proteins. The \( TbsMT \) mutant proteins catalysed zymosterol much less efficiently than the wild-type enzyme, with a \( V_{\text{max}}/K_{m} \) ratio 23–40% that of the parent strain (Table 3), yet there was no change in C-2-transfer activity. However, the sterol composition of the Y66F catalysis was different, appearing to derive from a change in the mono-alkylated sterol content; the sterol mixture possessed a significant decrease in ergosta-8,24(25)-dienol compensated by an increase in ergosta-8,25(27)-dienol and ergosta-8,24(28)-dienol (Supplementary Figure S6 at http://www.BiochemJ.org/bj/439/bj4390413add.htm). GC-MS analysis of the Y66F mutant did not show biosynthesis

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**Figure 4**  EI-MS (electrospray ionization-MS) spectra of \( TbsMT \) products

Products are from the incubation of cholesta-5,7,22,24-tetraenol paired with AdoMet (A) or [methyl-\(^{2}\)H\(_{3}\)]AdoMet (B). MS peak 3 is shown in Figure 3.

**Figure 5**  Postulated C-24-methylation pathways in the conversion of cholesta-5,7,22,24-tetraenol into \( \Delta^{22(28)} \)-olefin (path 1 to 1A to 2) and 22-hydroxy\( \Delta^{23(24)} \)-olefin (path 1 to 1A to 3) sterol side-chain constructions by \( TbsMT \).
Table 3 Comparison of kinetic constants and percentage distribution of ergostenols produced from zymosterol by wild-type and tyrosine to phenylalanine residue TbSMT mutant proteins

Rates are the averages of duplicate assays, using the same lysate preparation for all three substrates, performed under standard incubation conditions for 1 h. Results were fitted non-linearly to the Michaelis–Menten equation using the enzyme kinetic module (SPSS); accordingly, the mean standard error of the V_{max} determinations was ± 3 pmol/min · mg⁻¹ and r² = 0.95. For enzyme-generated product analysis identical assay conditions were used except that the assay continued for 16 h. Values in parentheses are the catalytic competence normalized to 100 % in reference to zymosterol or wild-type.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>V_{max} (pmol · min⁻¹ · mg⁻¹)</th>
<th>K_{m} (µM)</th>
<th>V_{max}/K_{m}</th>
<th>Ergosta-8, 25 (27)-dienol</th>
<th>Ergosta-8, 24 (28)-dienol</th>
<th>Ergosta-8, 24 (25)-dienol</th>
<th>24-Dimethyl-ergost-8, 25 (27)-dienol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>67</td>
<td>22</td>
<td>3.04 (100)</td>
<td>50</td>
<td>13</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>Y66F</td>
<td>19</td>
<td>6</td>
<td>3.1 (102)</td>
<td>68</td>
<td>26</td>
<td>26</td>
<td>trace</td>
</tr>
<tr>
<td>Y177F</td>
<td>34</td>
<td>10</td>
<td>3.4 (112)</td>
<td>60</td>
<td>30</td>
<td>10</td>
<td>trace</td>
</tr>
<tr>
<td>Y208F</td>
<td>26</td>
<td>36</td>
<td>0.72 (24)</td>
<td>57</td>
<td>19</td>
<td>24</td>
<td>trace</td>
</tr>
</tbody>
</table>

**Table 6 Alignment of 24-SMT amino acid sequences of cloned 24-SMTs**

1. S. cerevisiae (ScSMT, GenBank® accession number NP_0131706); 2. Paracoccidioides brasiliensis (PbSMT, GenBank® accession number XM_002796521.1); 3. Pneumocystis carinii (PcSMT, GenBank® accession number Q96WX43); 4. Glycine max (GmSMT1, GenBank® accession number AAP04657); 5. Glycine max (GmSMT2, GenBank® accession number FJ483974); 6. Arabidopsis thaliana (AtSMT1, GenBank® accession number NP_001078579); 7. A. thaliana (AtSMT2, GenBank® accession number NP_173458); 8. T. brucei (TbSMT, GenBank® accession number AA42014). Regions identified as the sterol-binding segments are shown as reported in [43] for the Erg6p (S. cerevisiae 24-SMT). Light shaded colour represents conserved amino acid residues among 24-SMTs. Tyrosine residues considered to be part of the 24-SMT active site are dark shaded. 24-SMTs have different substrate specificities and, except for ScSMT1 and PbSMT1 which affect only the first C-1-transfer reaction, all other 24-SMTs in the Table are bifunctionally capable of catalysing either the first [Δ24-25]- or second [Δ24-28]- C-1-transfer reaction. The optimal substrate for each catalyst is reported: zymosterol (ZY), lanosterol (LA), cycloartenol (CA) and 24(28)-methylene lophenol (ML) and their structures are shown in Supplementary Figure S1 at http://www.BiochemJ.org/bj/439/bj4390413add.htm.

**Figure 6 Alignment of 24-SMT amino acid sequences of cloned 24-SMTs**

The AdoMet- (SAM; magenta) and sterol- (STE; orange) binding pockets are shown. Tyrosine residues that were substituted are labelled in blue. Relevant contact amino acids identified in the homology modelling are shown in stick representation. The image was using PyMol with coordinates obtained by homology modelling against CMA coordinates as described in Materials and methods section.

**Figure 7 Ribbon representation of the active site of TbSMT**

The AdoMet- (SAM; magenta) and sterol- (STE; orange) binding pockets are shown. Tyrosine residues that were substituted are labelled in blue. Relevant contact amino acids identified in the homology modelling are shown in stick representation. The image was using PyMol with coordinates obtained by homology modelling against CMA coordinates as described in Materials and methods section.

of 24-ethyl(idene) sterols nor was there any evidence that the second C-1-transfer activity involving ergosta-8,24(25)-dienol conversion into 24 dimethyl ergosta-8,25(27)-dienol was affected (Supplementary Figure S7 at http://www.BiochemJ.org/bj/439/bj4390413add.htm). Notably, ergosta-5,24-dienol tested with the mutant enzymes was shown to exhibit similar product profiles and catalytic competence to the wild-type enzyme (Supplementary Figures S7 and S8 at http://www.BiochemJ.org/bj/439/bj4390413add.htm), whereas ergosta-8,24(28)-dienol (fecosterol) was not a substrate for any mutant TbSMT proteins.

**DISCUSSION**

The major product of TbSMT catalysis is a 24β-methyl sterol [ergosta-8,25(27)-dienol] which, according to sterol chemotaxonomy, is a primitive compound. In the reaction mechanism leading to Δ(25(27))-olefins, a hydride ion migrates from C-24 to C-25 in a reversible manner which suggests that in both single- and double-transmethylation some stabilization is received by the consequent formation of a carbocation at C-25, implying that its C-24 carboxylation precursor is significantly long-lived and that the usual coupled methylation-deprotonation reaction is not concerted as it is for Erg6p [39,40,49,50]. The proton transfer catalysed by TbSMT occurs in such a way that the Z-methyl group of the zymosterol substrate is transformed into the S-methyl group of the product. This cis-selectivity in methyl to methylene elimination at C-25(27), previously noted in algal sterol biosynthesis [50], was confirmed by the 13C-labelling studies of the TbSMT product. In particular, methyl insertion and hydride migration are occurring on opposite faces of the original double bond as evidenced by the detection of the pro-R methyl group at C-25 in the formation of the minor TbSMT product, [27,13C]ergosta-8,24(28)-dienol.

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The formation of ergosta-8,24(28)-diene in the product set appears to be a more advanced trait since it is utilized in the biosynthesis of 24-ethylidene sterols on the path to 24e-ethyl sterols (sitosterol). However, the second alkylation of the Δ24(28) substrate was not carried out, rather ergosta-8,24(25)-diene was the alternative Δ24 substrate for the second C-1-transfer reaction yielding a 24-dimethyl sterol. The remarkable promiscuity in olefin product formation by TbSMT, compared with the other 24-SMTs studied to date, may reflect the inherent positional selectivity of the normal deprotonation step of the reaction [i.e. regiospecificity for deprotonation from the C-27 methyl (to vinyl) is favoured over C-28 methyl (to methylene) deprotonation]. As noted in studies of the deuterium isotope effects on TbSMT, a primary isotope effect on C-25 deprotonation was observed in ergosta-8,24(25)-diene formation, suggesting this deprotonation step is rate-limiting in the formation of 24-dimethyl sterols. By contrast, C-2-transfer activities catalysed by plant 24-SMTs and the Erg6p mutant proteins (Tyr81) are sensitive to proton loss from C-28, making this step rate limiting in Δ28 sterol formation [47,48,51–53].

Of the substrates tested in the present study, the only one that converted into an unexpected product was cholesta-5,7,22,24-tetraenol, the other Δ24 sterols converted into typical side-chain products formed by TbSMT. The tetraenol converted into two C-24 methyl sterols with the minor biomethyl product possessing the ‘native’ Δ22,24,28-diene structure and the major product possessing the previously uncharacterized Δ22,24,28,29-C-22-hydroxy group construction. In sterol structure–function tests of plant and fungal 24-SMTs, cholesta-5,7,22,24-tetraenol failed to bind productively. These results indicate that the conjugated Δ22,24,28,29-diene structure, which originates in the Erg6 mutant of defective 24-SMT, is not an acceptable substrate in the C-24-methylation reaction. Yet, Δ22,24,28,29 side chains occur naturally, as a result they are often considered to be intermediates on the path to 24β-alkyl sterols, such as ergosterol. It would appear that the order of enzymatic reactions to form the Δ22,24-β-methyl sterol side chain proceeds first by C-24 methylation to form the Δ22,24,28,29-olefin, then Δ22 desaturation followed by Δ24,28 reduction to form the 24β methyl group. A possible explanation for ordering of the C-24 methylation before Δ22 desaturation in the late stages of ergosterol biosynthesis may be due to the potential harmful effect of the intermediate C-22 cation species on 24-SMT activity. It is tempting to speculate that the Δ22,24-diene sterol could represent a substrate analogue, which upon C-24 methylation affords a ‘charged’ intermediate in a region of the protein that does not normally encounter reactive electrophilic centres and that therefore would be susceptible to alkylation. Indeed, we have shown the inactivation of 24-SMT syntheses by sterol analogues containing conjugated systems with a Δ24 bond due to attack of the resulting intermediate cations on sensitive nucleophilic groups of the enzyme which are in range of the rotationally flexible side chain; upon saponification of the enzyme preparation these intermediates sequester into the organic extracts as ‘diol’ structures possessing one hydroxy group at C-3 and the other hydroxy group at C-24 or C-26 [23,44] (Supplementary Figure S9 at http://www.BiochemJ.org/bj/439/bj4390413add.htm).

The multiple products generated by the protozoan TbSMT are different from those synthesized by plant 24-SMT1 or 24-SMT2, or the single product Erg6p, suggesting phyla-specific differences in the primary structural elements that determine product specificities. In AdoMet-dependent enzymes where the methyl transfer reactions proceed through electrophilic catalysis of an S,2 mechanism, the methyl cation is transferred to the double bond, forming a carbocation intermediate. The reaction course can proceed differently among 24-SMTs such that in some cases cyclopropane structures are formed along the lateral side chain [8,55]. The crystal structures of three CMAs from the bacterium M. tuberculosis have emerged to shed light on the location of the cofactor AdoMet in the active site of the protein [35,55,56]. We were intrigued by these findings, since the closest homologue of 24-SMT is CMA (16% identity and 28% similarity). As seen in the putative interactions between cofactor and active-site residues of CMA, several aromatic side chains were shown to align the active-site contour required to stabilize the carbocationic intermediate through cation π interactions, which is in agreement with our hypothesis for the topology of the 24-SMT active site. Homology modelling of TbSMT shows that zymosterol (the natural substrate) docked adjacent to AdoMet aligns near Trp310 (Trp325), Leu376 (Leu391) and His225 (His238) which correspond with sterol-recognition sites for Regions III and IV (Figure 7). These residues are likely to form a hydrophobic interaction with the acceptor from the α-face of the sterol and possibly hydrogen bond to the C-3 OH group. At the other end of the sterol, a set of residues form a core structure around the Δ24 double bond with Glu199 (Glu214), His194 (His199) and Tyr177 (Tyr192), corresponding with Region III, on top of the side chain and Tyr259 (Tyr264) and Tyr466 (Tyr471), corresponding with Region I, positioned below and towards the opposite face of the substrate double bond. From leucine residue screening mutagenesis experiments of Erg6p, His177 and Glu235 were shown to be essential to C-1-transfer activity, whereas replacement of Tyr310 and Tyr235 with leucine had no significant effect on zymosterol conversion into fecosterol; neither Tyr469 or Asp462 were studied since they are not absolutely conserved in this class of catalyst [25,27,45].

Consequently, when considering similarity of substrate interactions in the Michaelis complex of fungal, plant and protozoan 24-SMTs in which a common Δ24(28)-olefin is produced, we independently reasoned that the central region of these enzymes is lined with aromatic and acidic amino acid residues whose positions and orientation appear to be responsible for producing the specific geometry that determines the product specificity that occurs with each 24-SMT [42,43,45]. For the TbSMT, when the OH group of the aromatic residues located some distance away from the reactive carbocation intermediate at Tyr177 and Try208 are replaced with H (phenylalanine) catalytic activity decreases without a corresponding affect on the sterol composition in the mutant proteins. On the other hand, when Tyr466 located close to the substrate double bond is replaced with a phenylalanine residue the resulting activity in the mutant protein is markedly slowed accompanied by a modified C-1-transfer product profile, consistent with the proposed role and positioning of this residue in the activated complex.

The results of the present study and our earlier investigations have established that the active-site geometry of 24-SMTs is sufficiently versatile to direct the formation of different product sets, leading to suggestions of evolutionary relevance [16,45,48,50]. Perhaps most strikingly, deprotonations that form ergosta-8,25(27)-diene and ergosta-8,24(28)-diene involve protons chemically and geometrically distinct from that lost in the generation of ergosta-8,24(25)-diene, indicating that multiple amino acid residues could be acting as adventitious active-site bases on either side of the intermediate in the activated complex. These substrate novelties and single-residue modifications, strongly encourages crystallographic effort with 24-SMTs to more satisfactorily define contact amino acids that determine sterol compositions. The study of sterol methylating catalysts is a wide-open area that needs to be understood in more detail [57]. Indeed, the future of the enzymatic and biological research into 24-SMT will provide new insights into development of parasite-specific sterol biosynthesis inhibitors.
AUTHOR CONTRIBUTION
Jialin Liu performed steroid analysis, synthesis and purification of substrates/products. Kuluttungun Gunapathy and Chizaram Nwogwugwu performed the kinetic studies with the cloned 7αSMT. Ewa Wywial and Janusz Bujnicki developed the homology model of the 7αSMT. W. David Nes conceived the work and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Effect of substrate features and mutagenesis of active site tyrosine residues on the reaction course catalysed by *Trypanosoma brucei* sterol C-24-methyltransferase

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Figure S1  Natural sterol acceptors for 24-SMT

The acceptors are: zymosterol for fungal and protozoan 24-SMT1; lanosterol for fungal 24-SMT1; cycloartenol for plant 24-SMT1; and 24(28)-methylene lophenol for plant 24-SMT2 [1,2].

Figure S2  Time-course study of TbSMT incubated with saturating amounts of zymosterol and AdoMet

Zymosterol (100 μM) and AdoMet (100 μM) were diluted with catalytic amounts of [methyl-3H3]AdoMet in standard assay conditions as discussed in the Materials and methods section of the main text. When plotting the kinetics of TbSMT catalysis using the radioactivity in biomethyl product formation, the d.p.m. in product formation continued to increase after 60 min (by approximately 20% over the product formed at 60 min) and plateaus at 120–240 min of incubation, yet the specific activity (pmol·min⁻¹·mg⁻¹ of total protein) decreases with increasing time giving rise to the initial velocity conditions reported in the Materials and methods section of the main text.

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Figure S3  EI-MS spectra

Ergosta-8,25(27)-dienol (1), ergosta-8,24(28)-dienol (2), ergosta-8,24(25)-dienol (3) and 24-dimethyl ergosta-8,25(27)-dienol (4) from incubation of TbSMT with zymosterol paired with AdoMet (A), [27-13C]zymosterol paired with AdoMet (B) or [24-2H]zymosterol paired with AdoMet (C).
Figure S4  EI-MS spectra of TbSMT products generated from incubation of cholesta-5,7,24-trienol

Figure S5  500 MHz 1H-NMR spectrum of the TbSMT-generated product

Product was generated from incubation with cholesta-5,7,22,24-tetraenol and corresponds with the late-eluting GC peak at a RRTc value of 1.48 and a $M^+$ value of 412 a.m.u.

Figure S6  Sequence alignment comparison of CMA (PDB code IKP1) and T. brucei sterol C24-methyl transferase (TbSMT)

Cylinders represent $\alpha$-helices, arrows are $\beta$-stands. ss, secondary structure; ssp, secondary structure predictions.

Figure S7  GC-MS (total ion chromatogram) of incubations

The incubations shown are with wild-type TbSMT (A), zymosterol with Y66F TbSMT (B) and ergosta-5,24(25)-dienol with Y66F TbSMT (C).
Figure S8  EI-MS of incubations of Y66F TbSMT with ergosta-5,24(25)-dienol

Substrate (A) and product (B) from the incubation are shown. The GC profile can be seen in Figure S6(C).
Figure S9 Postulated C-24-methylation mechanisms involved in the formation of C-24 and C-26-hydroxylated side chains from sterol analogues proven to be suicide substrates

Reported in [3,4].

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