SUPPLEMENTARY ONLINE DATA

Characterization, Mutagenesis and Mechanistic Analysis of an Ancient Algal Sterol C24-Methyltransferase: Implications for Understanding Sterol Evolution in the Green Lineage

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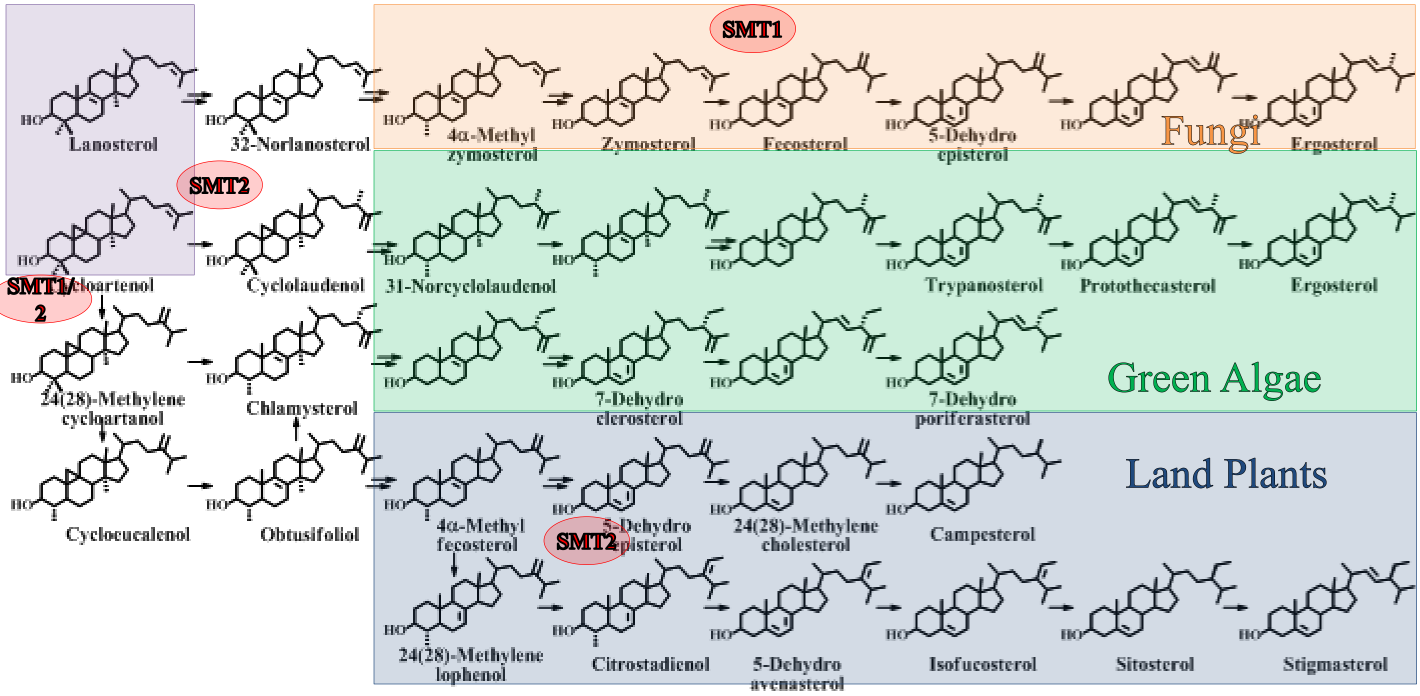
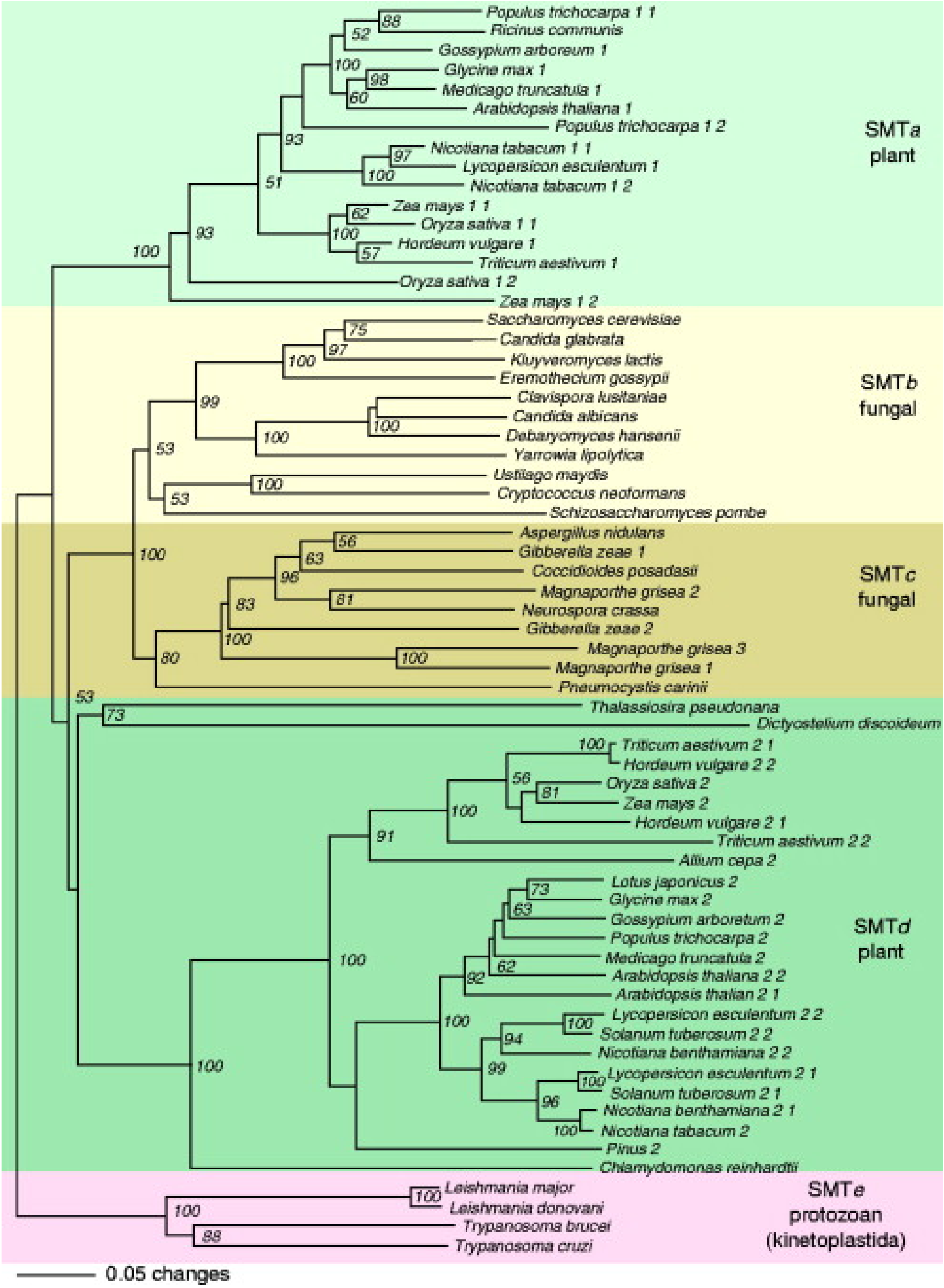


Supplemental Figure S1. Comparative sterol biosynthesis across kingdoms in Eukaryota as adapted from references 1-5. Biosynthetic steps catalyzed by SMTs are highlighted in red. .



Supplemental Figure S2. Phylogenetic tree of 24- and 28- SMTs. Adapted from References 3 and 5.

Supplemental Table S1. Comparison of the occurrence of putative and known SMT genes across kingdoms.

Strain[1]	**Class**	**Taxon**	**Source**[2]	**Subfamily**[3]	**Type**[4]	**Percent Similarity**[5]
Chlamydomonas reinhardtii	Chlorophyceae	Green alga	gi|158279461	d	SMT2	100%
Cucumis sativus	Magnoliopsida	Flowering plant	gi|449517755	a	SMT1-2	39.60%
Selaginella moellendorffii	Isoetopsida	Lycophyte	gi|302769362	a	SMT1	38.70%
Cucumis sativus	Magnoliopsida	Flowering plant	gi|449464010	a	SMT1	38.20%
Glycine max	Magnoliopsida	Flowering plant	gi|351725990	a	SMT1	37.10%
Physcomitrella patens	Bryopsida	Moss	gi|162662665	a	SMT1	35.80%
Amphimedon queenslandica	Demospongia	Demosponge	gi|340378018	a	SMT1	35.70%
Cyanidioschyzon merolae	Cyanidiophyceae	Red alga	gi|449018476	a	SMT1	30.10%
Aphanomyces euteiches	Oomycota	Water mold	gi|189026959	a	SMT1	27.60%
Saccharomyces cerevisiae	Saccharomycetes	Yeast	gi|396515	b	SMT1	37.10%
Paracoccidioides brasiliensis	Eurotiomycetes	Dimorphic fungus	gi|226288403	c	SMT1	34.50%
Cucumis sativus	Magnoliopsida	Flowering plant	gi|449437406	d	SMT2	48.90%
Physcomitrella patens	Bryopsida	Moss	gi|162676403	d	SMT2-2	48.80%
Physcomitrella patens	Bryopsida	Moss	gi|162676411	d	SMT2-1	48.10%
Selaginella moellendorffii	Isoetopsida	Lycophyte	gi|300156481	d	SMT2-1	48.10%
Physcomitrella patens	Bryopsida	Moss	gi|168047907	d	SMT2-3	47.70%
Selaginella. moellendorffii	Isoetopsida	Lycophyte	gi|300153528	d	SMT2-2	47.50%
Glycine max	Magnoliopsida	Flowering plant	gi|242755468	d	SMT2-2	46.00%
Glycine max	Magnoliopsida	Flowering plant	gi|242755433	d	SMT2-1	43.80%
Thalassiosira pseudonana	Coscinodiscophyceae	Diatom	gi|223996139	d	SMT2	38.00%
Thalassiosira pseudonana	Coscinodiscophyceae	Diatom	gi|224006796	d	SMT2	36.80%
Dictyostelium discoideum	Dictyostelia	Slime mold	gi|60464861	d	SMT2	35.90%
Ectocarpus siliculosus	Phaeophyceae	Brown algae	gi|299469830	d	SMT2	34.10%
Ectocarpus siliculosus	Phaeophyceae	Brown algae	gi|299469813	d	SMT2-1	32.20%
Trypanosoma cruzi	Zoomastigophora	Kinetoplastid	gi|407843552	e	SMT1	32.50%
Trypanosoma brucei	Zoomastigophora	Kinetoplastid	gi|70832598	e	SMT1	32.30%

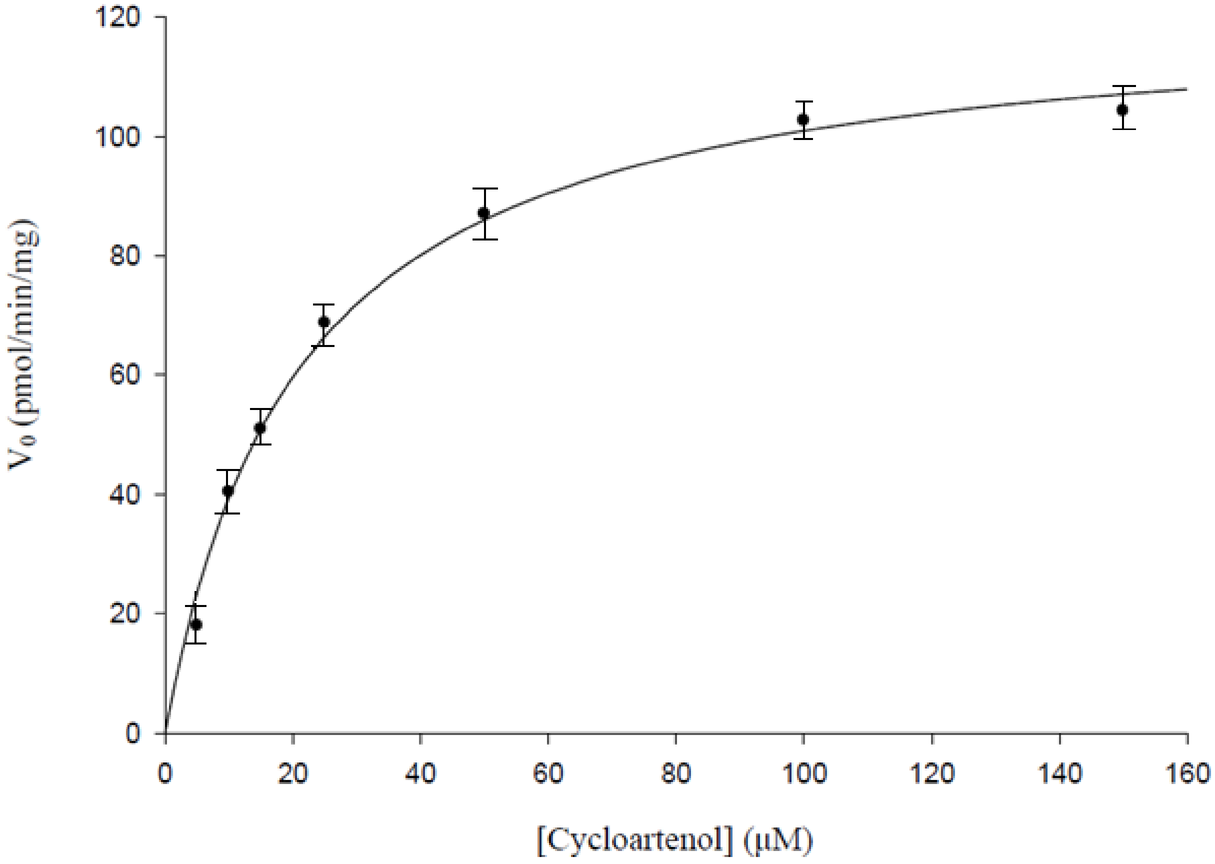
[1] Host organism of SMT

[2] Gene sequence from GenBank

[3] Based on the grouping of SMTs from the phyogenetic tree shown in Fig. S2.

[4] Based on the annotation from the GenBank or for *Cr*SMT and *E. siliculous* SMT2-1 based on biochemical reasoning,

[5] Percent similarity of amino acid sequences is relative to the amino acid sequence of *Cr*SMT.



Supplemental Figure S3. Michaelis-Menten plot determined under initial velocity conditions of *Cr*SMT2 incubated with cycloartenol at concentrations varied from 5 to 150 µM and SAM fixed at saturation of 100 µM . Reactions were performed for 45 min at 35 °C in phosphate buffer pH 7.5. Datapoints were assays of triplicate determinations. Error bars represent standard error (SEM).

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C. reinhardtii   SMT1 (1)     MAVALPAAVTSAYERLAGEFDKLSTTQKYAVGIAGGVTSLYLLAKVLKGSDRDKPTTL-----QLSGGSIDSSKVKDEFT   75
T. brucei        SMT1 (1)     -------------------MS------------AG---------------SRGPLSLLIARERDANGVNGDVNATAGRLR   34
S. cerevisiae    SMT1 (1)     -------------------MSETELRKRQAQFTRE----LHGD----DIGKKTGLSAL-----MSKNNSAQKEAVQKYLR   48
G. max           SMT1 (1)     -------------------MDPLSLFCTGALLAGG----LYWFVCVLGPAEQKGKRAT-----DLSGGSISAEKVQDNYK   52
A. thaliana      SMT2 (1)     -------------------MDSLTLFFTGALVAVG----IYWFLCVLGPAERKGKRAV-----DLSGGSISAEKVQDNYK   52

C. reinhardtii   SMT1 (76)    AYADSYGKNAGEGITDRSKTVHLVDVFYSLVTDIYEWGWGQSFHFSPKLPNKDLKASEAAHEARIAALLRLQPGQKALDC   155
T. brucei        SMT1 (35)    DRYDGKGASAS---ERRQDATSLTNEYYDIVTDFYEYGWGQNFHFAPRYMNETFYESLARYEYFLAYHAQFKPTDTVLDV   111
S. cerevisiae    SMT1 (49)    NWDGRTDKDAE--ERRLEDYNEATHSYYNVVTDFYEYGWGSSFHFSRFYKGESFAASIARHEHYLAYKAGIQRGDLVLDV   126
G. max           SMT1 (53)    QYWSFFRRPKE--IETADKVPDFVDTFYNLVTDIYEWGWGQSFHFSPSIPGKSHRDATRLHEEMAVDLIEAKPGNRILDV   130
A. thaliana      SMT2 (53)    QYWSFFRRPKE--IETAEKVPDFVDTFYNLVTDIYEWGWGQSFHFSPSIPGKSHKDATRLHEEMAVDLIQVKPGQKILDV   130

C. reinhardtii   SMT1 (156)   GCGVGGEMRTVAAVSGAHITGITINQYQVDRAKTHNARQGLAPLTDVVRGDFTNMPFKENTFDGAYAIEATCHAPKLEQV   235
T. brucei        SMT1 (112)   GCGIGGEARNMVRFTSCNVMGVNNNEYQINRARQHDSRYGMSGKINYTKTDFCNMCFGDNEFDGAYAIEATCHSESKVKC   191
S. cerevisiae    SMT1 (127)   GCGVGGEAREIARFTGCNVIGLNNNDYQIAKAKYYAKKYNLSDQMDFVKGDFMKMDFEENTFDKVYAIEATCHAPKLEGV   206
G. max           SMT1 (131)   GCGVGGEMRAIAAHSRANVVGITINEYQVNRARMHNKKAGLESLCEVVCGNFLKMPFPDNSFDGAYSIEATCHAPKLEEV   210
A. thaliana      SMT2 (131)   GCGVGGEMRAIASHSRANVVGITINEYQVNRARLHNKKAGLDALCEVVCGNFLQMPFDDNSFDGAYSIEATCHAPKLEEV   210

C. reinhardtii   SMT1 (236)   YGEIYRVLKPGSYFVSYEWVSTQKFDVNNAEHVKIMDEINFGNGLPEMRTWKEAEDAGKNVGFELVMSLDLATA----SV   311
T. brucei        SMT1 (192)   YSEVFRAIKPGAYFMLYEWCLTDLYDPANEEHQRVRHGIELGDGLPELDTMRQVVAAVKAAGFVVEESFDMAERFESGEP   271
S. cerevisiae    SMT1 (207)   YSEIYKVLKPGGTFAVYEWVMTDKYDENNPEHRKIAYEIELGDGIPKMFHVDVARKALKNCGFEVLVSEDLADN-----D   281
G. max           SMT1 (211)   YAEIFRVLKPGALYVSYEWVTTDKYRGDDPEHVEVIQGIERGDALPGLRNYTDIAETARKVGFAVVKERDLAKP-----P   285
A. thaliana      SMT2 (211)   YAEIYRVLKPGSMYVSYEWVTTEKFKAEDDEHVEVIQGIERGDALPGLRAYVDIAETAKKVGFEIVKEKDLASP-----P   285

C. reinhardtii   SMT1 (312)   VAGPWY-----------------ERLRMGKYTHAINHGIVSTVDALGLAPKGLKEVHHMLVEVAKSLIQGGESGIFTPMH   374
T. brucei        SMT1 (272)   KSVPWYEPLQGSY-------TSLSGLRATPAGRWLTSVTCRLLEAVRLAPAGTCKATEILEEGAVNLVKGGELGIFTPSF   344
S. cerevisiae    SMT1 (282)   DEIPWYYPLTGEWKYVQNLANLATFFRTSYLGRQFTTAMVTVMEKLGLAPEGSKEVTAALENAAVGLVAGGKSKLFTPMM   361
G. max           SMT1 (286)   AQ-PWW-----------------SRLKMGRIAYWRNHIVVTVLAALGIAPKGTVDVHEMLFKTADYLTRGGDSGIFSPMH   347
A. thaliana      SMT2 (286)   AE-PWW-----------------TRLKMGRLAYWRNHIVVQILSAVGVAPKGTVDVHEMLFKTADYLTRGGETGIFSPMH   347

C. reinhardtii   SMT1 (375)   LLLFRKPGADKKK---------   387
T. brucei        SMT1 (345)   FVKARKPRLGEELSC-------   359
S. cerevisiae    SMT1 (362)   LFVARKPENAETPSQTSQEATQ   383
G. max           SMT1 (348)   MILCRKPHDKDDHN--------   361
A. thaliana      SMT2 (348)   MILCRKPESPEESS--------   361
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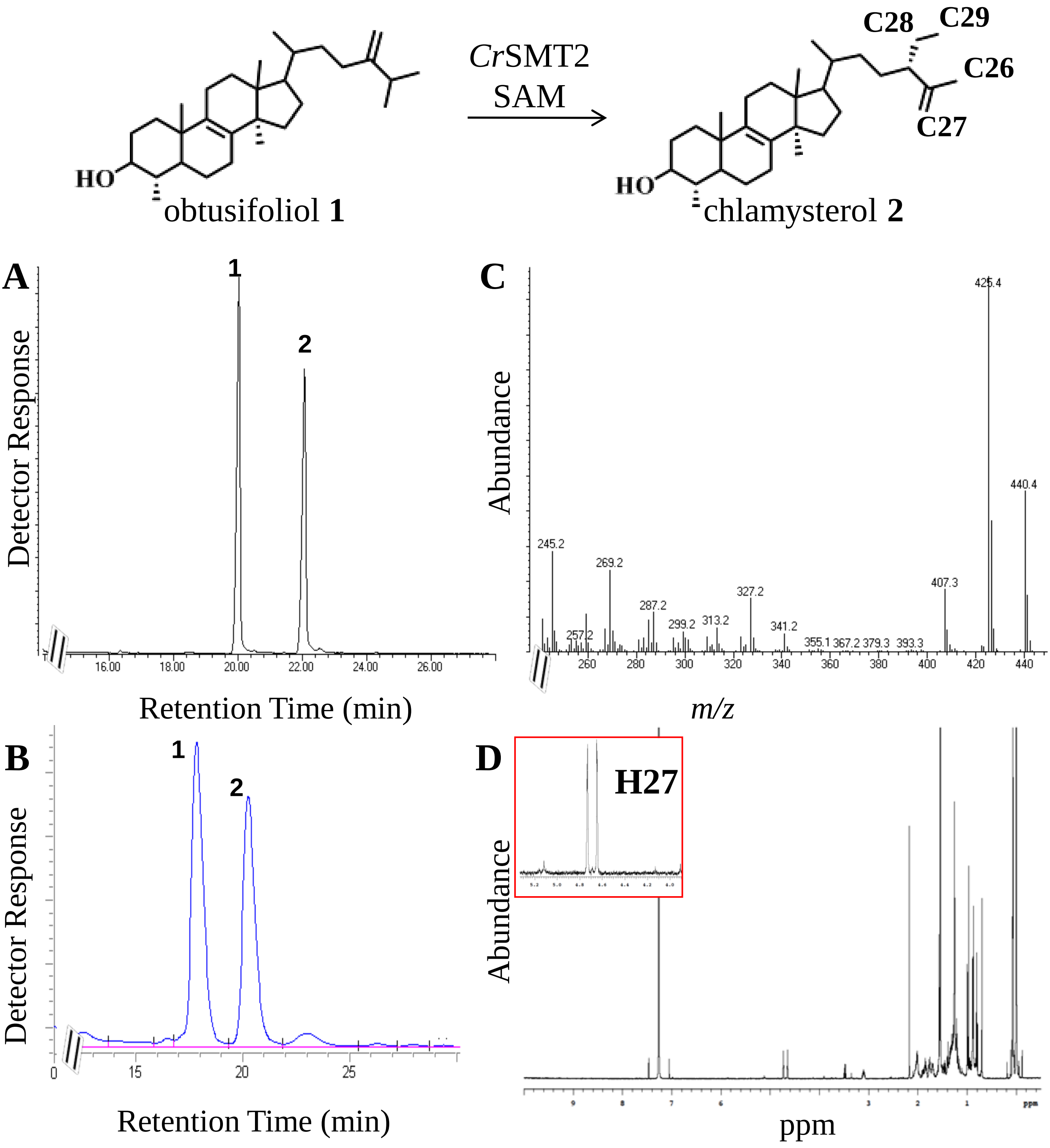
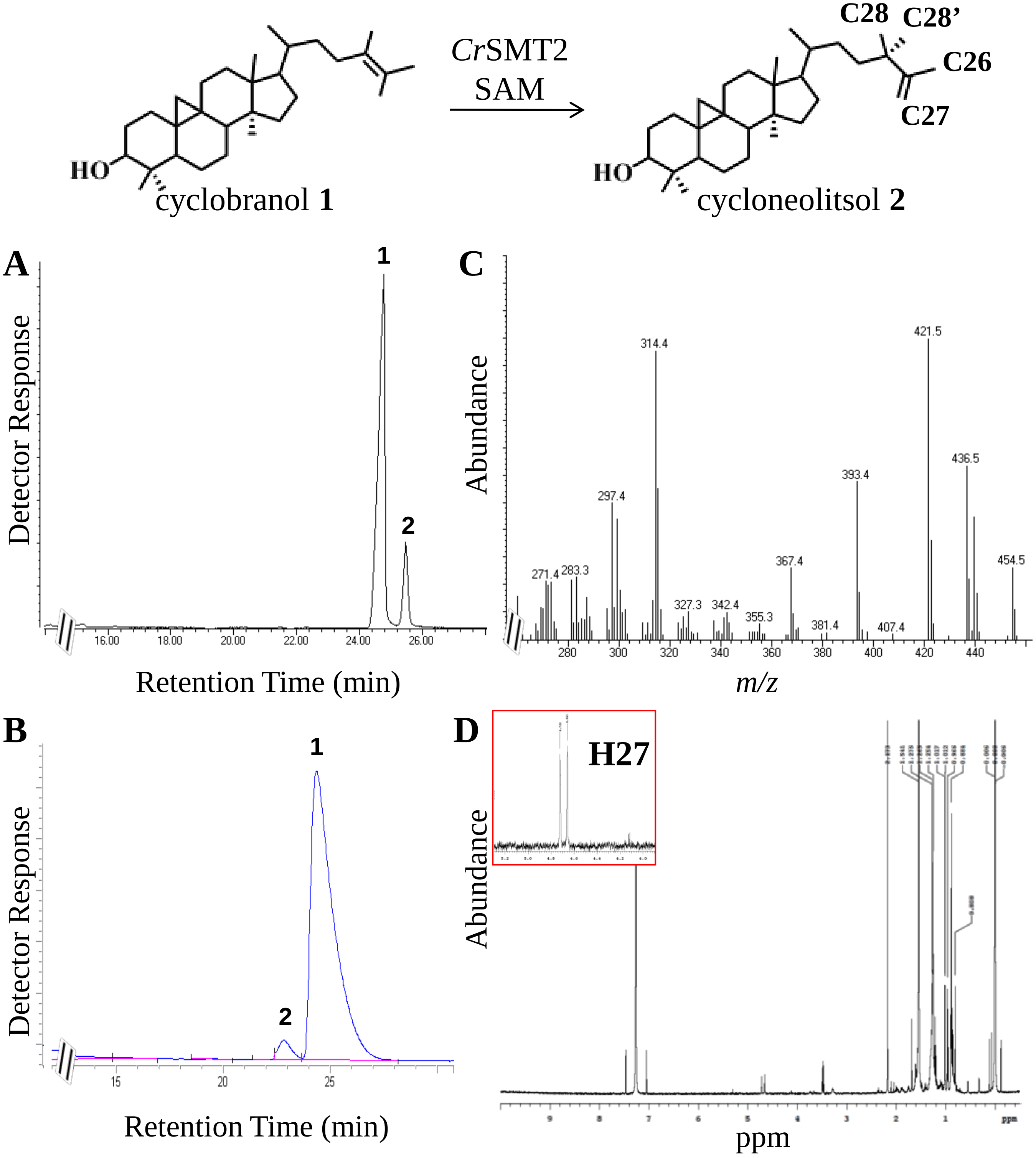
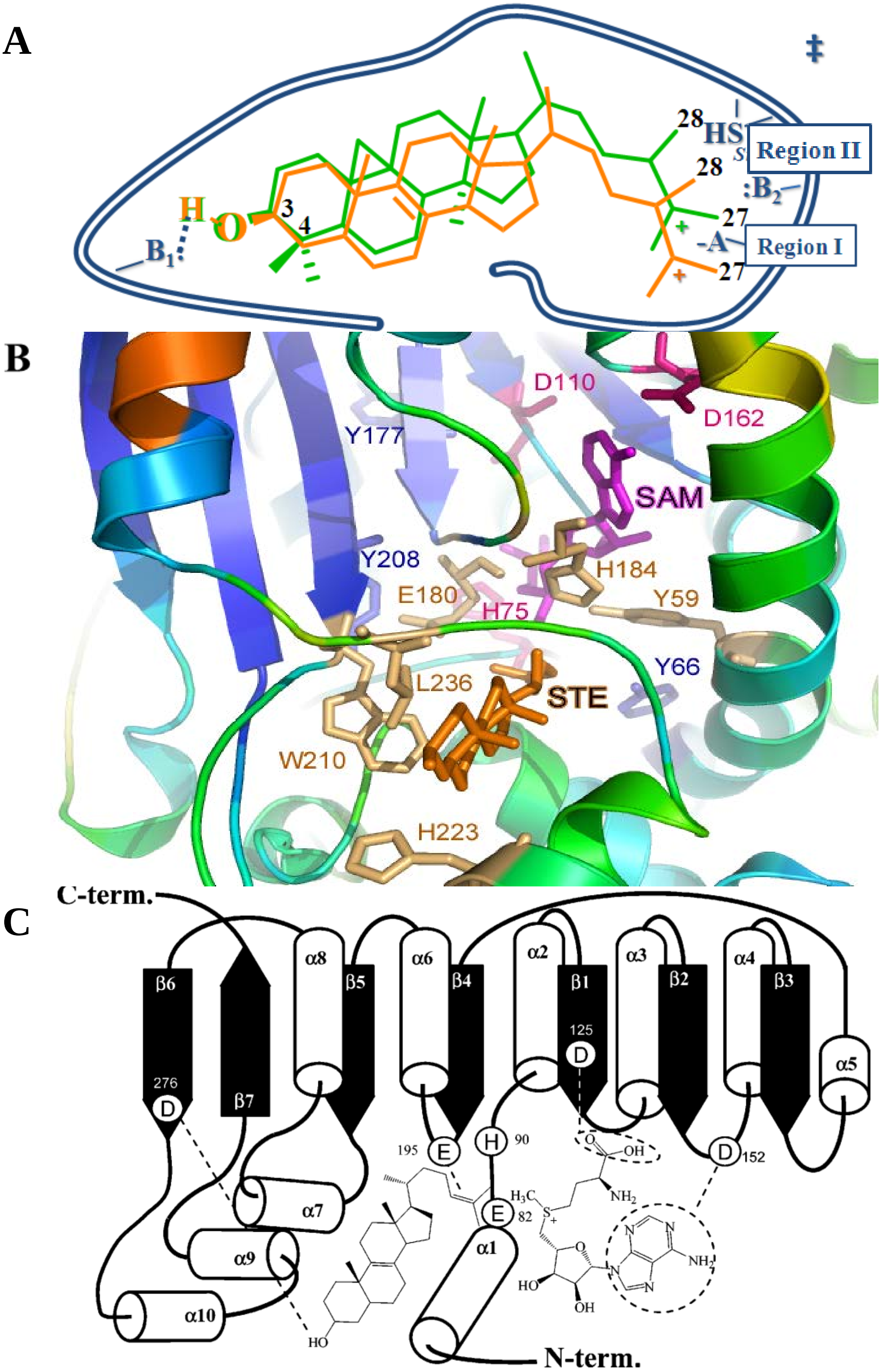
Supplemental Figure S4. Alignment of sterol methyltransferase amino acid sequences (GenBank accession numbers) from *C. reinhardtii* (gi|159465129), *T. brucei* (gi|70832598), *S. cerevisiae* (gi|6323635), *G. max* (gi|242755433) and *A. thaliana* (gi|332191841). Identical residues conserved in the primary structure are in red. The sequences were aligned using Align X (Informax Inc) with defauled parameters. The deduced substrate preference of SMT catalyzes the first (Δ24(25)-substrate or second (Δ24(28)-substrate) C1-transfer reaction, SMT1 or SMT2 is reported. Sterol and SAM binding sites are indicated as boxed Regions 1, 2 and 4 or Region 2, respectively.



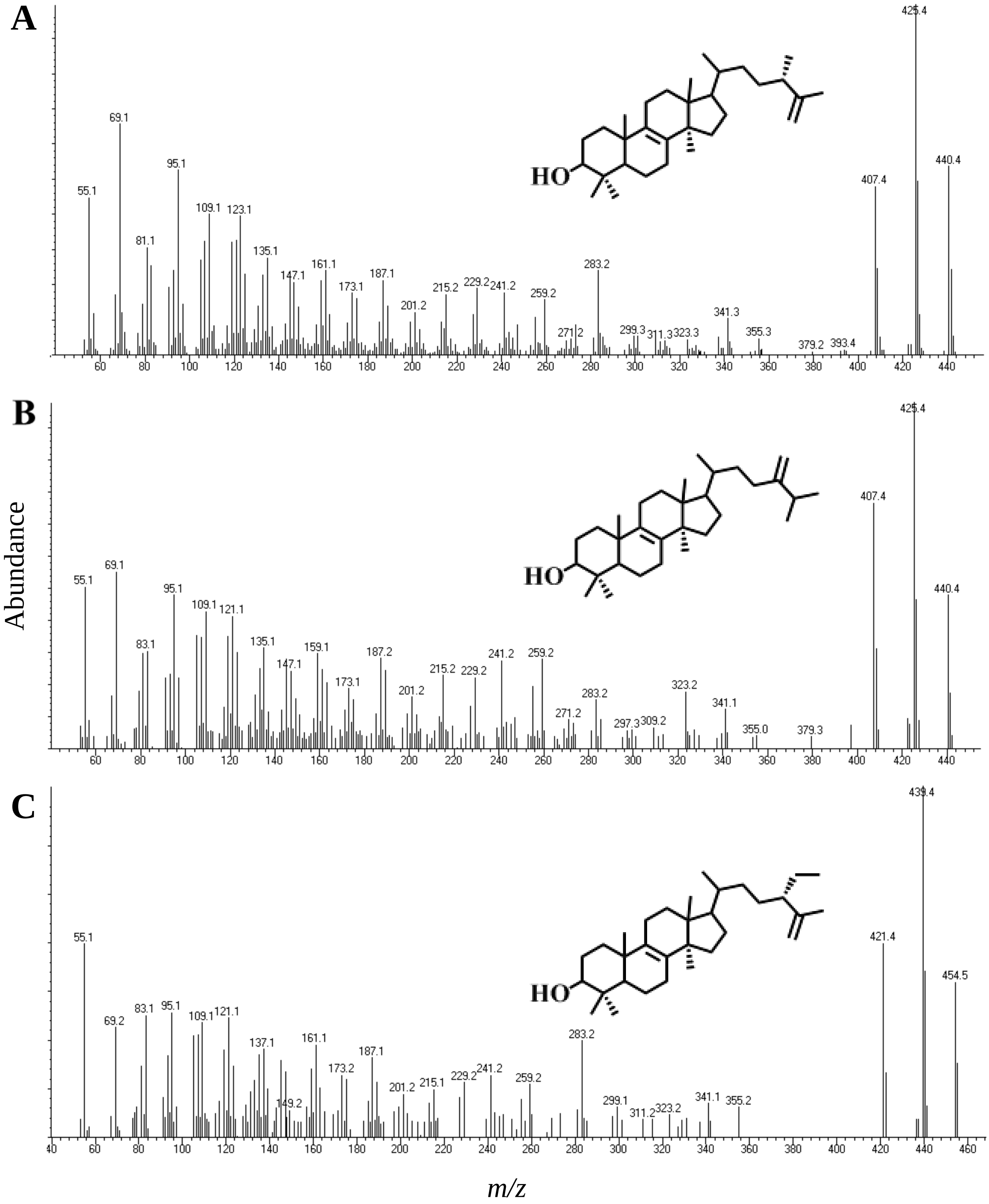
Supplemental Figure S5. Spectral and chromatographic properties of *Cr*SMT2-generated chlamysterol 2. (A) TIC chromatogram of obtusifoliol 1 conversion to chlamysterol 2. C-MS (30 m HP-5 capillary column) coupled to HP 6890 gas chromatograph interfaced to a 5973 mass spectrometer at 70 eV; GC flow rate of He was set at 1.2 ml/min, injector port was 250 ^{o}C and the initial temperature was set at 170 ^{o}C, held for 3 min, and increased at 19 ^{o}C/min to 280 ^{o}C. (GC Method B) Cholesterol eluted at 16.5 min. (B) RP-HPLC chromatogram of 1 conversion to 2. Phenomenex Luna C18(2) column, 250 mm x 4.6 mm x 5 µm, (Phenomenex, Torrance) eluted with 100 % methanol at 1 mL/min and 20 °C, monitored at 210 nm. At these conditions, cholesterol eluted at 17 min. (C) High-end EI mass spectrum of 2. Base peak is 425 [M^+ – Me]. (D) ^{1}HNMR at 500 MHz of 2. Olefinic signals of H27.



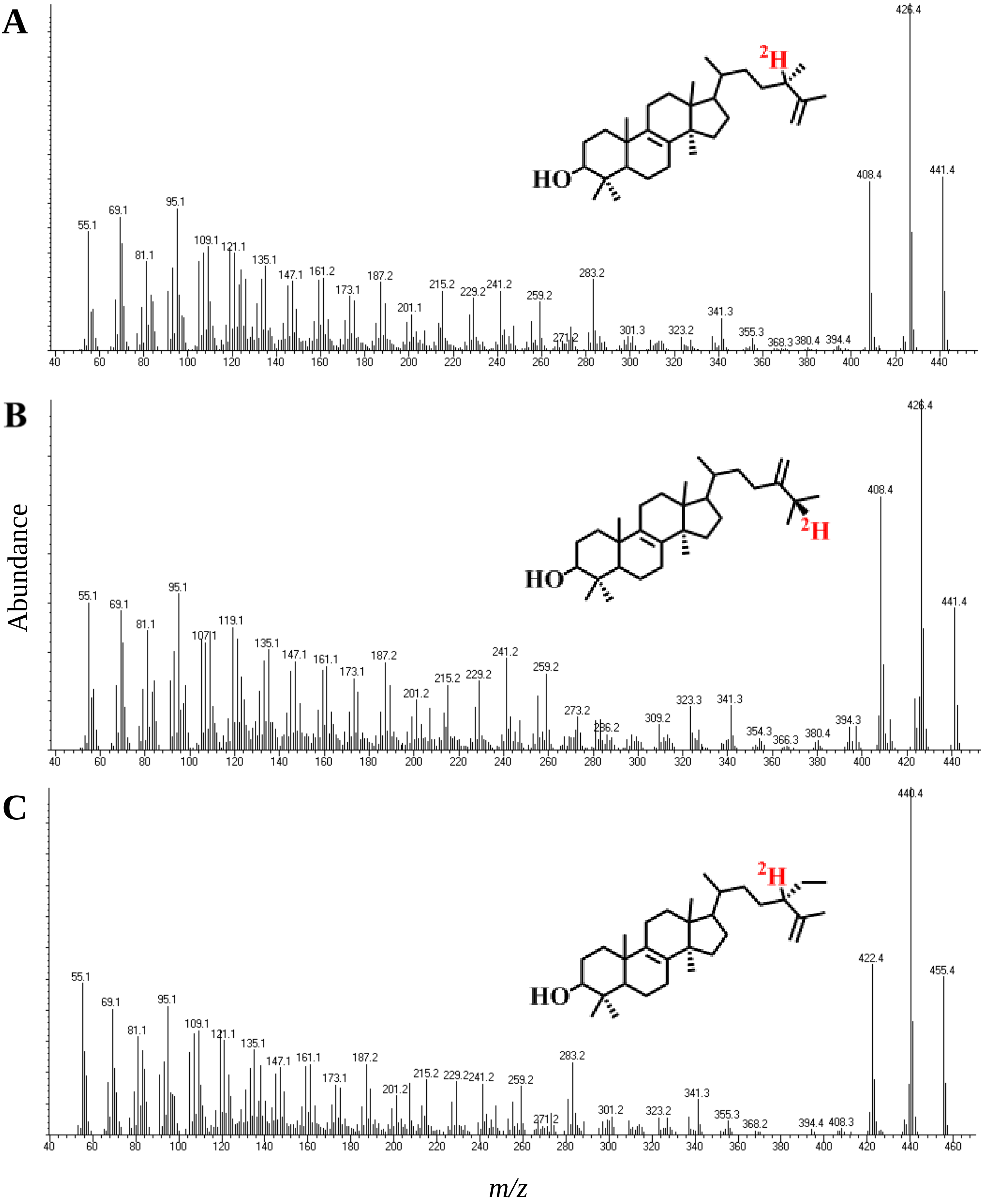
Supplemental Figure S7. Spectral and chromatographic properties of *Cr*SMT-generated cycloneolitsol. (A) TIC chromatogram of cyclobranol 1 conversion to cycloneolitsol 2. GC flow rate of He was set at 1.2 ml/min, injector port was 250 °C and the initial temperature was set at 170 °C, held for 3 min, and increased at 20°C/min to 280 °C. Cholesterol eluted at 14.2 min. (B) RP-HPLC chromatogram of 1 conversion to 2. Phenomenex Luna C18(2) column, 250 mm x 4.6 mm x 5 um, eluted with isocratic 60:40 (v/v) acetonitrile/2-propanol at 1 ml/min and 25 °C, monitored at 210 nm. Cholesterol eluted at 12 min. (C) High-end EI mass spectrum of 2. High end has been adjusted to show detail (base peak is 95 amu, M^{+} is 4 %, M^{+} - Pr 421 is 17 %). (D) ^{1}HNMR at 500 MHz of 2. Inset: Olefinic signals of H27.



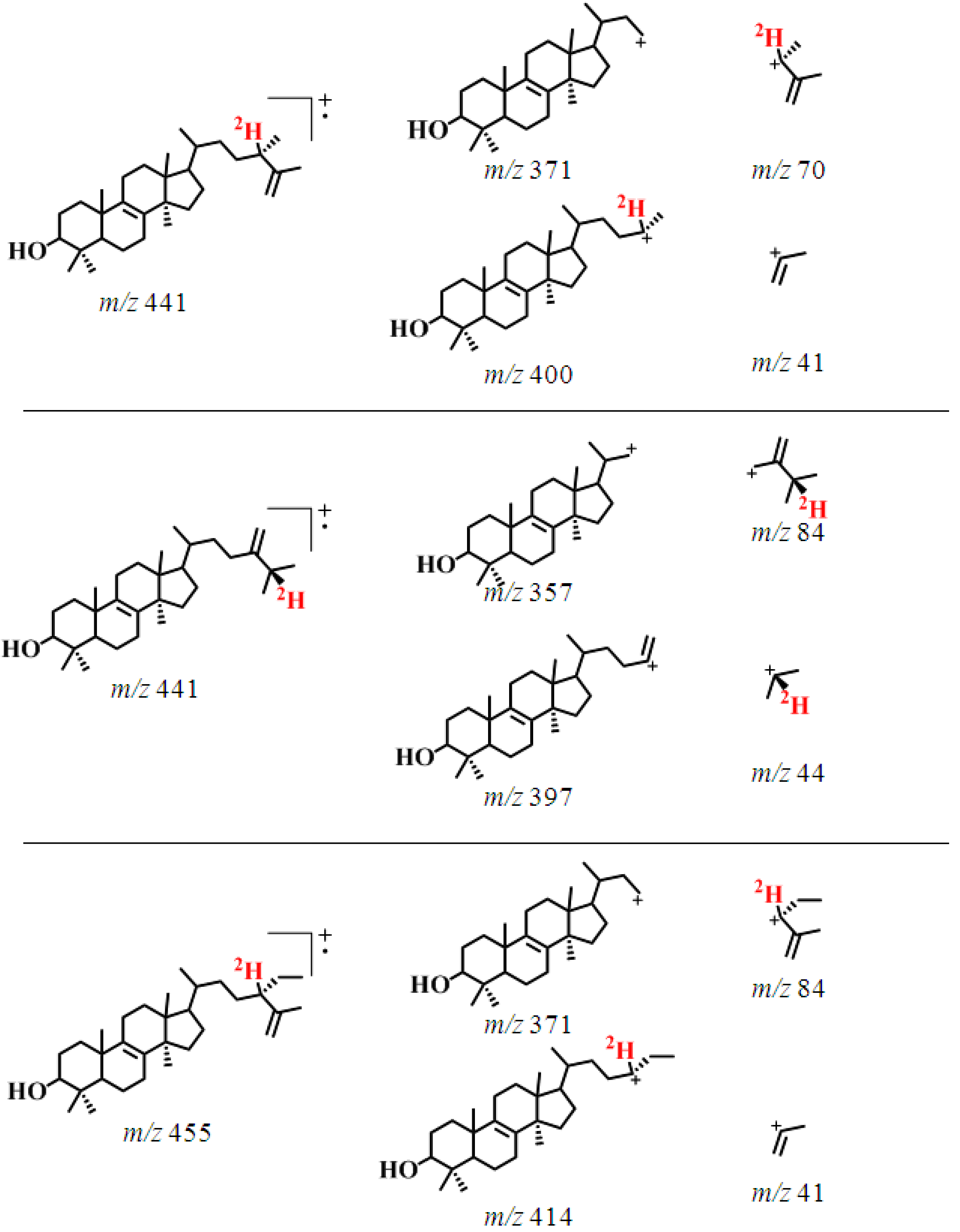
Supplemental Figure S7. Different views of substrate-SMT interactions (adapted from references 5-7): A- 24-methylated zymosterol (orange) and 24-methylated cycloartenol (green) bound in the transition state, B and C-Homology models are taken from *T*bSMT and *Sc*SMT, respectively , where details of the modeling are described including software. .



Supplemental Figure S8. Mass spectra of products of first and second C_1-transfer to lanosterol by *Cr*SMT2. A. 24β-methyl lanosta-8,25(27)-dienol B. eburicol (24(28)-methylenelanost-8-enol) C. 24β-ethyll anosta-8,25(27)-dienol . (see text for details for preparations of activity assays).



Supplemental Figure S9. Mass spectra of products of first and second C_1-transfer to [24-^{2}H]lanosterol by *Cr*SMT2. A. 24β-methyl [24α-^{2}H]lanosta-8,25(27)-dienol B. [25-^{2}H]eburicol (24(28)-methylene [25-^{2}H]lanost-8-enol) C. 24β-ethyl [24-^{2}H]lanosta-8,25(27)-dienol



Supplemental Figure S10. Molecular ions and diagnostic fragmentations for stereospecifically labeled products of first and second C_1-transfer to [24-^{2}H]lanosterol by *Cr*SMT2 . EI-mass spectral data was collected from 50-550 a.m.u.

REFERENCES

1. Weete, J. D., Abril, M. and Blackwell, M. (2010) Phylogenetic distribution of fungal sterols. Plos One 5(5):e10899

2. Miller, M. B., Haubrich, B. A., Wang, Q., Snell, W. J. and Nes, W. D. (2012) Evolutionary conserved $\Delta^{25(27)}$-olefin ergosterol biosynthesis pathway in the alga *Chlamydomonas reinhardtii*. J. Lipid Res. **53**, 1636-1645

3. Neelakandan, A. K., Song, Z., Wang, J., Richards, M. H., Wu, X., Valliyodan, B., Nguyen, H. T. and Nes, W. D. (2009) Cloning, functional expression, and phylogenetic analysis of plant 24C-methyltransferases involved in sitosterol biosynthesis. Phytochemistry **70**, 1982-1998

4. Goad, L. J. and Akihisa, T. (1997) Analysis of sterols. Blackie Academic, London

5. Zhou, W., Nguyen, H. T. and Nes, W. D. (2008) Plant sterol methyltransferases: phytosterolomic analysis, enzymology, and bioengineering strategies. *In* Methods in Plant Biochemistry and Molecular Biology Vol. 1. N. G. Lewis, editor. Elsevier Science, Amsterdam. 241-281

6. Ganapathy, K., Jones, C. W., Stephens, C. M., Vatsyayan, R., Marshall, J. A. and Nes, W. D. (2008) Molecular probing of the *Saccharomyces cerevisiae* sterol C24- methyltransferase (SMT) reveals multiple amino acids involved in C2-transfer activity. Biochim. Biophys Acta **1781,** 344-351

7. Liu, J., Ganapathy, K. , Wywial, E., Bujinicki, J. M., Nwogwugwu, C.A. and Nes, W. D. (2011) Effect of substrate features and mutagenesis of active site tyrosine residues on the reaction course catalyzed by *Trypanosoma brucei* sterol C24-methyltransferase. Biochem. J. **439,** 413-422