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CLONING AND CHARACTERIZATION OF THE *ARABIDOPSIS THALIANA* LUPEOL SYNTHASE GENE

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Abstract—A 2274 bp *Arabidopsis thaliana* cDNA was isolated that encodes a protein 57% identical to cycloartenol synthase from the same organism. The expressed recombinant protein encodes lupeol synthase, which converts oxidosqualene to the triterpene lupeol as the major product. Lupeol synthase is a multifunctional enzyme that forms other triterpene alcohols, including β-amyrin, as minor products. Sequence analysis suggests that lupeol synthase diverged from cycloartenol synthase after plants diverged from fungi and animals. This evolutionary order is the reason that fungi and animals do not make lupeol.

INTRODUCTION

Thousands of nonsteroidal triterpenes have been identified and the ubiquity of these compounds among higher plants implies that they are essential. However, the function of the nonsteroidal triterpenes remains unclear. The cyclization, rearrangement and deprotonation reactions mandated by the biogenetic isoprene rule are well-established [1–4], but it is unknown how triterpene synthases mediate the specific bond changes, or how these enzymes evolved. Obtaining triterpene synthase genes is crucial to resolving these problems in terpene biosynthesis. If triterpene synthase genes from a model plant were known, transgenic plants with modified triterpene production could be made and their phenotypes studied. Correlating catalytic activity with sequences from multiple triterpene synthases would allow identification of candidate catalytic residues. Phylogenetic trees constructed with orthologous and paralogous triterpene synthases would illuminate the evolutionary relationships between these enzymes. With these goals in mind, we have undertaken a program to characterize genes encoding triterpene synthases in a model organism, the plant *Arabidopsis thaliana*.

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RESULTS AND DISCUSSION

Although *A. thaliana* has not been reported to produce nonsteroidal triterpenes, DNA sequence databases contain clues that this plant may cyclize oxidosqualene to at least one compound in addition to cycloartenol. A search for potential oxidosqualene cyclases in the GenBank database of randomly sequenced partial cDNA fragments uncovered a short *A. thaliana* sequence [5] (accession No. T22249) encoding a 39-residue peptide 61% identical to a portion of *A. thaliana* cycloartenol synthase (CAS1) [6]. This fragment was sequenced as part of the Arabidopsis Genome Project [5] and although it was clearly related to cycloartenol synthase, it seemed too far diverged to represent a second isozyme that produced cycloartenol. A full-length cDNA corresponding to this fragment was sought by using the fragment to probe an *A. thaliana* cDNA plasmid library [7]. One hybridizing plasmid (pJR1) with an insert the same size (2.5 kbp) as *A. thaliana CAS1* was analyzed in detail.

The pJR1 insert was sequenced (the sequence has been deposited in the GenBank database, accession No. U49919) and a yeast expression plasmid was constructed as described in the experimental section. Recombinant yeast transformed with this plasmid acquired the ability to convert exogenous oxidosqualene to the pentacyclic triterpene alcohol

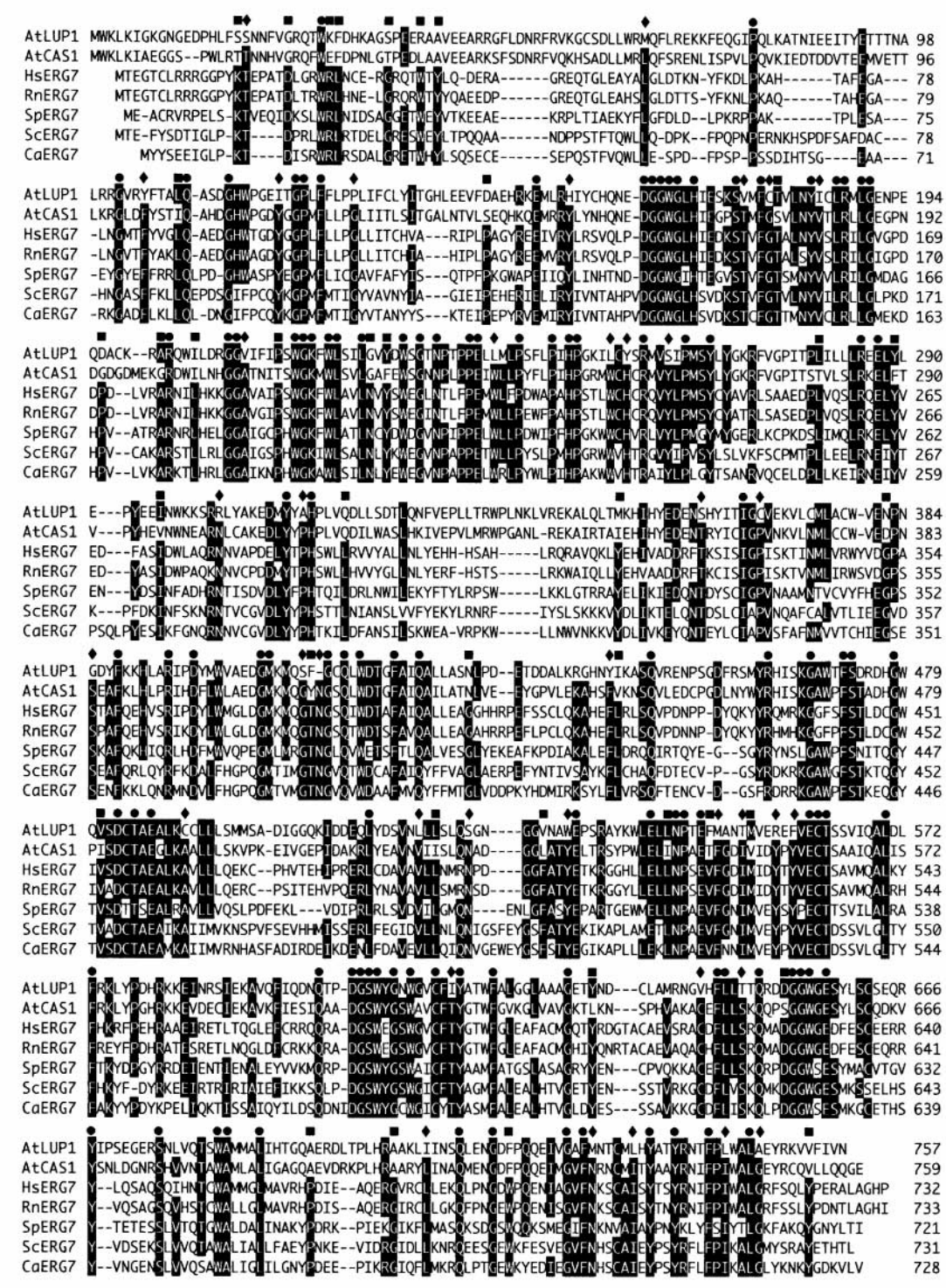

Fig. 1. Sequence comparison of oxidosqualene cyclases. Alignments of the deduced amino acid sequences of *A. thaliana* lupeol synthase (AtLUP1) and *A. thaliana* cycloartenol synthase (AtCAS1) [6] with lanosterol synthases from human (HsERG7) [15, 16], rat (RnERG7) [13, 14], *Schizosaccharomyces pombe* (SpERG7) [17], *Saccharomyces cerevisiae* (ScERG7) [11, 12] and *Candida albicans* (CaERG7) [9, 10]. Sequences were aligned with the Megalign program (DNAStar) using the Clustal method. Amino acid residues identical in at least five of the seven sequences are boxed and hyphens indicate gaps introduced to maximize alignments. Residues identical in all seven oxidosqualene cyclases are indicated by ●, those identical in the protosteryl-cation cyclases by ◆ and those identical in the lanosterol synthases by ■.

Table 1. Percent amino acid identity between oxidosqualene cyclase enzymes

	AtLUP1	AtCAS1	PsCAS1	HsERG7	RnERG7	SpERG7	ScERG7	CaERG7
AtLUP1	100	57	57	37	37	35	33	32
AtCAS1	57	100	82	43	43	38	36	34
PsCAS1	57	82	100	44	43	40	36	34
HsERG7	37	43	44	100	85	45	42	42
RnERG7	37	43	43	85	100	46	42	41
SpERG7	35	38	40	45	46	100	46	47
ScERG7	33	36	36	42	42	46	100	64
CaERG7	32	34	34	42	41	47	64	100

Percent similarities were calculated with the Megalign program (DNAStar). Abbreviations are as in Fig. 1 with the addition of *Pisum sativum* cycloartenol synthase (PsCAS1) [8].

lupeol. The enzyme also produced minor amounts of β-amyrin and other unidentified triterpene alcohols. The new gene therefore encodes lupeol synthase and was named *LUP1*. A sequence alignment of lupeol synthase with *A. thaliana* cycloartenol synthase and the known lanosterol synthases is shown in Fig. 1. The LUP1 enzyme is the first oxidosqualene cyclase shown to make multiple products.

The amino acid sequences of *A. thaliana* cycloartenol synthase and lupeol synthase are 57% identical, but those of the known *A. thaliana* and pea cycloartenol synthases [6, 8] and the lanosterol synthases [9–17] from various species are only 34–44% identical (Table 1). This is superficially counterintuitive, since cycloartenol synthase and lanosterol synthase (which initially cyclize to the protosteryl cation, Fig. 2) are mechanistically more closely related to one another than either is to lupeol synthase (which initially cyclizes to the dammarenyl cation). Apparently, cycloartenol synthase and lanosterol synthase diverged relatively long ago

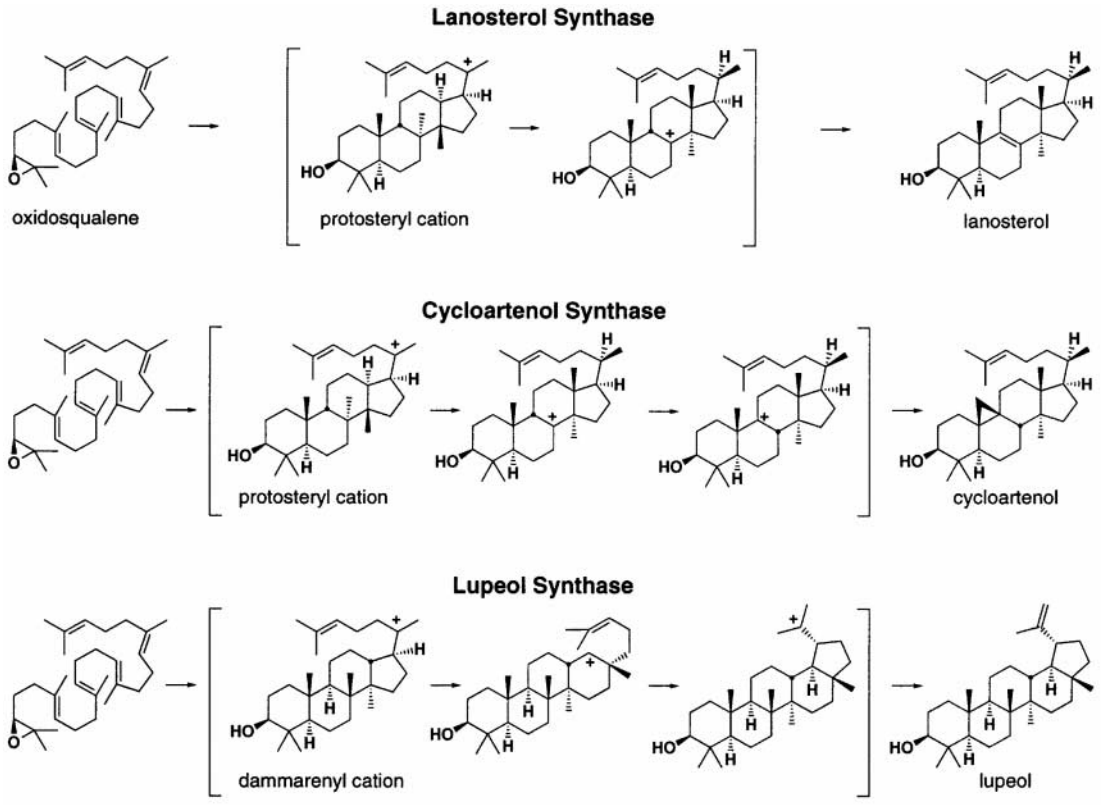

Fig. 2. Mechanisms of cloned triterpene synthases. Lanosterol synthase cyclizes oxidosqualene to lanosterol via the protosteryl cation [20]. Cycloartenol synthase cyclizes oxidosqualene to cycloartenol via the same intermediate carbocation [19]. Lupeol synthase cyclizes oxidosqualene to lupeol via the dammarenyl cation [2]. The conformations drawn do not represent energy minima, but those that occupy the least space.

and accumulated mutations at most noncatalytic residues, but have undergone constant selection to maintain catalytic specificity for sterol ring systems. The overall sequence similarity of lupeol and cycloartenol synthases evidently results from their recent divergence. The numerous conserved residues (very few of which should be catalytic, since only the A ring is formed identically in cycloartenol and lupeol biosynthesis) outnumber the catalytic residues that have changed to alter the structure of the product. Since overall sequence similarity does not correlate with catalytic similarity, it cannot be relied upon as a tool for predicting which structures will result from newly cloned oxidosqualene cyclase genes.

Overall sequence predicts tertiary structure similarity by the expression Δ (Å) = $0.4e^{1.87H}$, where Δ is the root mean square deviation of main chain atom positions (in Å) and H is the fraction of mutated residues [18]. The 57% identity between the *A. thaliana* lupeol synthase and cycloartenol synthase suggests that the tertiary structures of these enzymes are so similar that lupeol synthase residues are on average located within an angstrom of their cycloartenol synthase counterparts. Oxidosqualene cyclases apparently share a common architecture; differences in the amino acids that constitute their active sites are probably responsible for differences in product structures.

Candidate catalytically relevant residues can be identified by correlating conserved residues with the catalytic events that these enzymes promote. Lupeol synthase, cycloartenol synthase and lanosterol synthase open the epoxide ring to form identical A rings (Fig. 2). If the residues that perform these tasks are identical in all of these enzymes, they are among those marked with a circle in Fig. 1. After A ring formation, lupeol synthase follows a course distinct from the other two enzymes. Mechanistic studies have shown that cycloartenol synthase and lanosterol synthase create the BCD ring fusions similarly to form the protosteryl cation [19, 20]. Both enzymes then guide 1,2 shifts in the BCD rings to form the lanosteryl cation. Stereochemical features of lupeol suggest that lupeol synthase cyclizes oxidosqualene to the dammarenyl cation (Fig. 2), which undergoes ring expansion to the baccharenyl cation, followed by annulation to the lupenyl cation and deprotonation to lupeol [2, 4]. Cycloartenol synthase and lanosterol synthase should therefore share active site residues responsible for BCD ring formation and these should differ from the residues at the same positions in lupeol synthase. Candidate residues for forming the BCD rings in cycloartenol synthase and lanosterol synthase are marked with a diamond. The lanosterol synthase residues that deprotonate the lanosteryl cation from C-9 should differ from cycloartenol synthase, which deprotonates at C-19, and are among those at positions marked with a square. The recently solved squalene–hopene cyclase crystal structure [21] provides structural information about a related active site. Lupeol synthase residues that are active-site candidates by the above criteria and that align with proposed squalene cyclase active site residues are: I120, F125, P129, P215, W217, W221, Y257, S262, I366, S410, F411, W416, F472, V481, D483, W532, E548, M550, T553, W610, Y616, F726, L732, Y734, Y737. This prediction may be compromised by structural differences between oxidosqualene cyclases and squalene cyclases or by unreliable alignment in regions with low similarity. This set of candidates is unusually rich in aromatic amino acids, which may serve to stabilize intermediate carbocations through cation-π interactions [9, 22].

Enzymes that convert oxidosqualene to other pentacyclic triterpenes should be reasonably similar to lupeol synthase. These enzymes should be accessible by a PCR-based strategy using primers that correspond to sequences conserved between cycloartenol synthase and lupeol synthase. Additional homologs will illuminate structure–function relationships and evolutionary relationships of these enzymes. As the number of known sequences increases, the candidates for active site residues should decrease, thereby facilitating the testing of mechanistic theories by site-directed mutagenesis. The roles that triterpenes play in plants is poorly understood, but should be addressable by producing transgenic plants with altered triterpene synthase activities and examining the resulting phenotypes.

EXPERIMENTAL

Cloning the LUP1 *gene*

The GenBank database (http://www.ncbi.nlm.nih.gov/Recipon/index.html) was searched using the BLAST algorithm [23] for sequences similar to *A. thaliana* cycloartenol synthase. Plasmid 103D18T7 appeared to be related and was obtained from the Arabidopsis Biological Resource Center at The Ohio State University. The insert was excised from 103D18T7 with *Not*I and *Sal*I and labeled with α-^{32}P-dCTP using random hexamer primers [24]. The radiolabeled probe was used to screen an *A. thaliana* cDNA plasmid library [7] by colony hybridization [24].

Plasmid characterization

Plasmid DNA from sixteen positives was purified and cDNA sizes were estimated by digesting each clone with *Not*I (which cuts on both sides of the insert) and comparing fragment electrophoretic mobility with standards on agarose gels [24]. One plasmid with an insert the same size as the cyclo-

artenol synthase cDNA (2.5 kbp) was named pJR1 and analyzed in detail. The *Not*I fragment containing the cDNA insert was purified from an agarose gel and a sequencing plasmid was constructed by ligating the *Not*I-excised insert to pBluescript II KS(+) (Stratagene) cut with the same enzyme. Fragments were ligated using T4 DNA ligase and *E. coli* strain DH5α was transformed with the ligation mixtures [24]. Plasmid DNA from the resultant transformants was mapped with restriction enzymes and subclones for sequencing were made by digesting with enzymes that cut both the insert and the polylinker, purifying the vector-containing DNA fragment, recircularizing this DNA by ligation and transforming *E. coli* strain DH5α. DNA from these subclones was sequenced using the Applied Biosystems International Automated Sequencer and the dye primer method with PBST3 and EXT7 primers as recommended by the manufacturer. The reported sequence was determined at least once on each strand.

Expression of LUP1

The *S. cerevisiae* lanosterol synthase mutant SMY8 (*MAT***a** *erg7::HIS3 hem1::TRP1 ura3-52 trp1-Δ63 leu2-3,112 his3-Δ200 ade2* Gal^+) [17] was the expression host. The high-copy expression vector pRS426GAL was a gift from Ling Hua (Rice University) and consists of the pRS316GAL [25] promoter sequence in pRS426 [26]. pJR1.16, a *URA3*-marked 2μ (high copy) plasmid encoding *A. thaliana* lupeol synthase under galactose control, was constructed by inserting the *Xba*I–*Sac*I fragment of JR1 into pRS426GAL cut with *Xba*I and *Sac*I. Yeast lanosterol synthase auxotroph SMY8 was transformed with pJR1.16 using LiOAc [24] and transformants were selected on synthetic complete media plates (2% glucose and 1.5% agar) lacking uracil and supplemented with ergosterol (20 μg/ml, supplied from a stock of 100 mg ergosterol in 50 ml 1:1 EtOH/Tween 80) and heme (13 μg/ml, supplied from a stock of 65 mg heme in 50 ml 1:1 EtOH/10 mM NaOH).

Enzymatic synthesis and identification of lupeol from S. cerevisiae *expressing* A. thaliana LUP1

SMY8 cells transformed with pJR1.16 were cultured in synthetic complete medium containing 2% glucose and lacking uracil and supplemented with ergosterol and heme as above. The cells were grown and induced in five 2 l Erlenmeyer flasks, each of which was inoculated with 200 ml of saturated recombinant yeast culture and 1 l of the same growth medium, except that the sugar source was 2% galactose. After shaking at 250 rpm for 24 h at 30°C, 54 g yeast cells were collected by centrifugation at 5000*g* for 20 min and suspended in 140 ml 500 mM Na–Pi buffer at pH 5.6. The large inoculum and short induction time were essential; smaller inocula and longer induction times produced little enzyme activity. The reaction conditions were those found to be optimal for lanosterol synthase [27] and activity or product distribution might be changed by varying pH or salt composition.

The suspension was lysed in a French Press at 20,000 psi and 14 ml (±)-2,3-oxidosqualene [28] solution (20 mg/ml in 20% Triton X-100) and an additional 140 ml 500 mM Na–Pi buffer at pH 5.6 was added to give final concentrations of 1% Triton X-100 and 1 mg/ml oxidosqualene. After 24 h at 25°C, 600 ml EtOH was added to terminate the reaction. The denatured protein precipitate was removed by filtration and the volume was halved by rotary evaporation. Silica gel (20 g) was added to the flask and the remaining solvent was removed by rotary evaporation. The dry silica was added to a 2.5×20 cm column and the lipids were eluted with 600 ml Et_2O. This workup procedure was used because the Triton X-100 made aqueous extraction difficult. The eluate was dried over Na_2SO_4, concentrated by rotary evaporation and purified by silica gel chromatography (10% Et_2O in hexane) to yield 116 mg of a fraction that comigrated with triterpene alcohols on silica gel TLC ($R_f = 0.56$ in 1:1 hexane–Et_2O).

A sample of the crude product (3 mg) was acetylated and GC analysis (30 m × 0.25 mm Restek Rtx-5 WCOT capillary column, 0.1 μm film thickness; 1:40 split; isothermal at 280°C; FID at 290°C; inlet at 280°C; He carrier gas at 24.7 ml/min) showed peaks corresponding to lupeol acetate and β-amyrin acetate in a ratio of 4:1, as well as three other unidentified products. A second sample of lupeol was prepared similarly and purified twice by silica gel chromatography (the first eluted with 5% Et_2O in hexane and the second with 25% CH_2Cl_2 in hexane). This procedure afforded 27 mg lupeol that was ~70% pure by ^1H and ^{13}C NMR (~38% yield based on (*S*)-oxidosqualene). One of the minor triterpene components was identified as β-amyrin based on a comparison with NMR signals of an authentic standard (a gift from Professor E. J. Corey, Harvard University). Thirty resolved minor ^{13}C NMR signals matched those of the standard to ±0.01 ppm and six resolved minor ^1H NMR methyl and olefinic signals matched to ±0.001 ppm. The minor signals were *ca.* 10–15% of the intensity of the lupeol signals. A *ca.* 95% pure sample (1 mg) of the major product obtained by $AgNO_3$–silica gel (1:9) chromatography [29, 30] was shown to be lupeol by ^1H NMR, COSYDEC, HSQC, IR and MS analyses, which gave spectra identical within experimental error to those obtained from an authentic sample (Sigma), as did a ~70% pure sample by ^{13}C NMR spectroscopy: ^1H NMR (500 MHz, 5 mM in $CDCl_3$, 25°C) δ 0.683 (*m*, 1H, H-5α), 0.761 (*s*, 3H, H-24), 0.788 (*t*, $J = 0.8$ Hz, 3H, H-28), 0.829 (*d*, $J = 1.0$ Hz, 3H, H-25), 0.903 (*tdd*,

$J = \sim 13.0$, 4.2, 0.9 Hz, 1H, H-1α), 0.945 (*d*, $J = 1.0$ Hz, 3H, H-27), 0.968 (*s*, 3H, H-23), 1.003 (*ddd*, $J = \sim 13.5$, 4, 3 Hz, 1H, H-15α), 1.031 (*s*, 3H, H-26), ~1.065 (*m*, 1H, H-12α), 1.191 (*dddd*, $J = \sim 12$, 11, 10, 0.9 Hz, 1H, H-22α), ~1.220 (*m*, 1H, H-11β), ~1.272 (*m*, 1H, H-9α), 1.323 (*dddd*, $J = 13.6$, 10.2, 5.8, 1.3 Hz, 1H, H-21α), 1.361 (*t*, $J = \sim 11.7$ Hz, 1H, H-18α), 1.371 (*td*, $J = \sim 13.2$, 4.4 Hz, 1H, H-16α), 1.383 (*dd*, $J = \sim 11.8$, 8.7 Hz, 1H, H-22β), 1.39 (*m*, 3H, H-6β, H-7α, H-7β), 1.412 (*m*, 1H, H-11α), 1.478 (*ddd*, $J = 12.9$, 4.6, 2.6 Hz, 1H, H-16β), 1.516 (*m*, 1H, H-6α), 1.555 (*tdd*, $J = \sim 13.2$, 11.6, 3.9 Hz, 1H, H-2β), 1.609 (*br dq*, $J = \sim 12.8$, 4.3 Hz, 1H, H-2α), ~1.659 (*m*, 1H, H-13β), ~1.665 (*m*, 1H, H-12β), 1.668 (*dt*, $J = \sim 13.1$, 3.6 Hz, 1H, H-1β), 1.681 (*dd*, $J = 1.4$, 0.8 Hz, 3H, H-30), 1.682 (*td*, $J = \sim 13.6$, 5.0 Hz, 1H, H-15β), 1.918 (*dddd*, $J = 13.6$, 10.8, 10.0, 8.3 Hz, 1H, H-21β), 2.378 (*td*, $J = 11.1$, 5.8 Hz, 1H, H-19β), 3.188 (*dd*, $J = 11.4$, 4.9 Hz, 1H, H-3α), 4.566 (*dq*, $J = 2.5$, 1.4 Hz, 1H, H-29E), 4.687 (*dt*, $J = 2.5$, 0.7 Hz, 1H, H-29Z); ^{13}C NMR (125 MHz, 50 mM in $CDCl_3$, 25°C) δ 14.56, 15.38, 15.99, 16.13, 18.01, 18.33, 19.32, 20.94, 25.15, 27.43, 27.45, 28.00, 29.86, 34.29, 35.59, 37.18, 38.06, 38.72, 38.87, 40.01, 40.84, 42.84, 43.01, 48.00, 48.31, 50.45, 55.31, 79.01, 109.33, 150.98; IR (film) 3365, 2940, 2854, 1637, 1465, 1453, 1380, 1043, 1037, 1013, 983, 883 cm^{-1}; MS (EI): *m*/*z* (rel. int) 426 ($[M]^+$,38), 393 (100), 365 (6), 336 (5), 279 (6), 218 (28), 207 (32), 189 (34); HRMS (EI), $[M]^+$ *m*/*z* calcd for $[C_{30}H_{50}O]^+$: 426.3862, found: 426.3863.

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