Supplementary Information

Kilo-scale GMP Synthesis of Renewable Semisynthetic Vaccine-Grade Squalene.

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GENERAL

Biological origination of Amyris farnesene produced by fermentation and the renewable nature of semi-synthetic squalene

As envisioned both RSMs would derive from fermentation-produced *trans*-β-farnesene[1], ensuring the sustainability of the end product. The renewable carbon content of squalane made from the same fermentation-derived *trans*-β-farnesene used here has been reported. [2] As both the ASTM D6866-12 Standard Test Methods for Determining the Biobased Content of Solid, Liquid, and Gaseous Samples Using Radiocarbon Analysis or the $^{13}C/^{12}C$ isotope ratio method only focus on the carbon backbone, they both give 100% results. Strictly speaking, and based on the reaction mechanisms involved, the only non-biogenic atom in the squalene molecule is the hydride that replaces the sulfone group in the very last step. This comes presumably from the

borohydride reducing agent used. Accounting for this the resulting squalene would be 99.75% renewable (one non-biogenic hydrogen in the $C_{30}H_{50}$ molecular formula).

Analytical method development

During the early phases of this work, where the established European Pharmacopoeia (EP) 2020: 2085 GC test method for the quantitation of squalene method was used for monitoring the synthesis steps and assaying the purity of the squalene target, it became apparent that the monograph method was inadequate for correctly checking this chemistry. This is illustrated in Figure S1 and Figure S2, where in the first, a comparison of the overlaid GC traces obtained using the monograph method for an official EP reference standard sample of squalene and a sample of the new semi-synthetic squalene, one may observe that in addition to several new poorly resolved minor impurities eluting after the squalene peak, the latter also displays evident asymmetry on the front and tail end, suggesting the possible presence of hidden coeluting species. Should this be the case the monograph method would be overestimating the squalene content. This problem was addressed by the development of a high-resolution GC-FID method (GC-IPC) employing a higher resolution GC column with the same stationary phase and by switching to a gradient elution profile rather than the monograph isothermal conditions. With this change the asymmetry resolves into two distinct peaks, as illustrated in Figure S2.

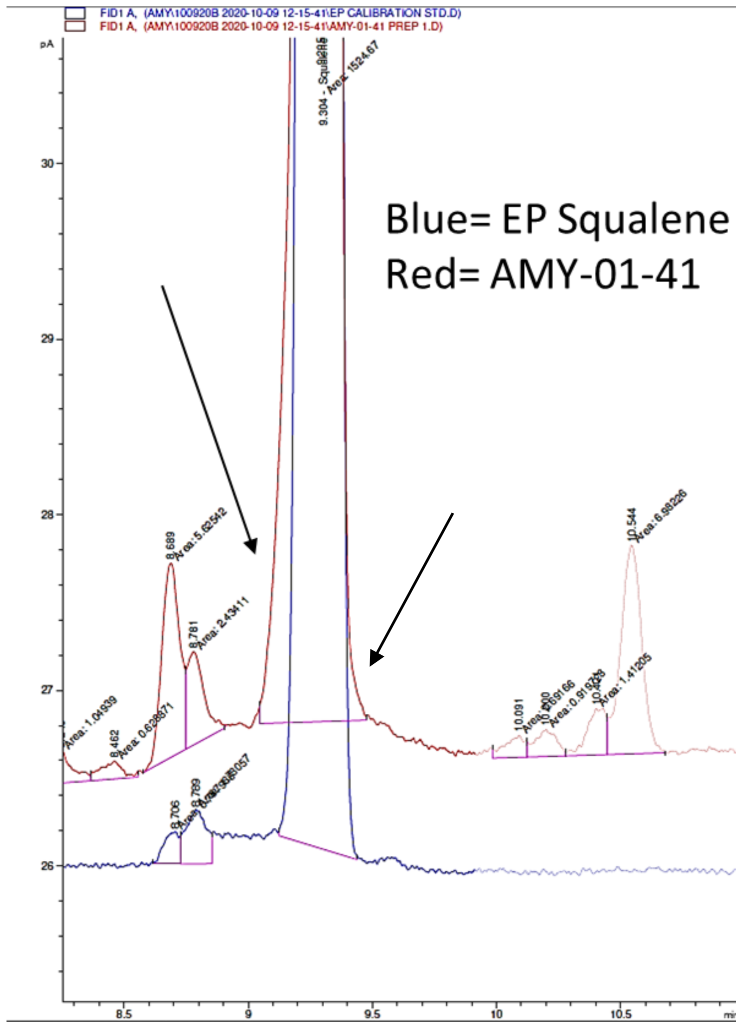


Figure S1: Comparison of EDQM reference squalene standard (blue trace) with a sample of semi-synthetic squalene (red trace) assayed using the European Pharmacopoeia 10.0 squalene monograph (01/202:2805) gas chromatography method. Arrows indicate the front and tail shoulders observed for the semi-synthetic squalene peak.

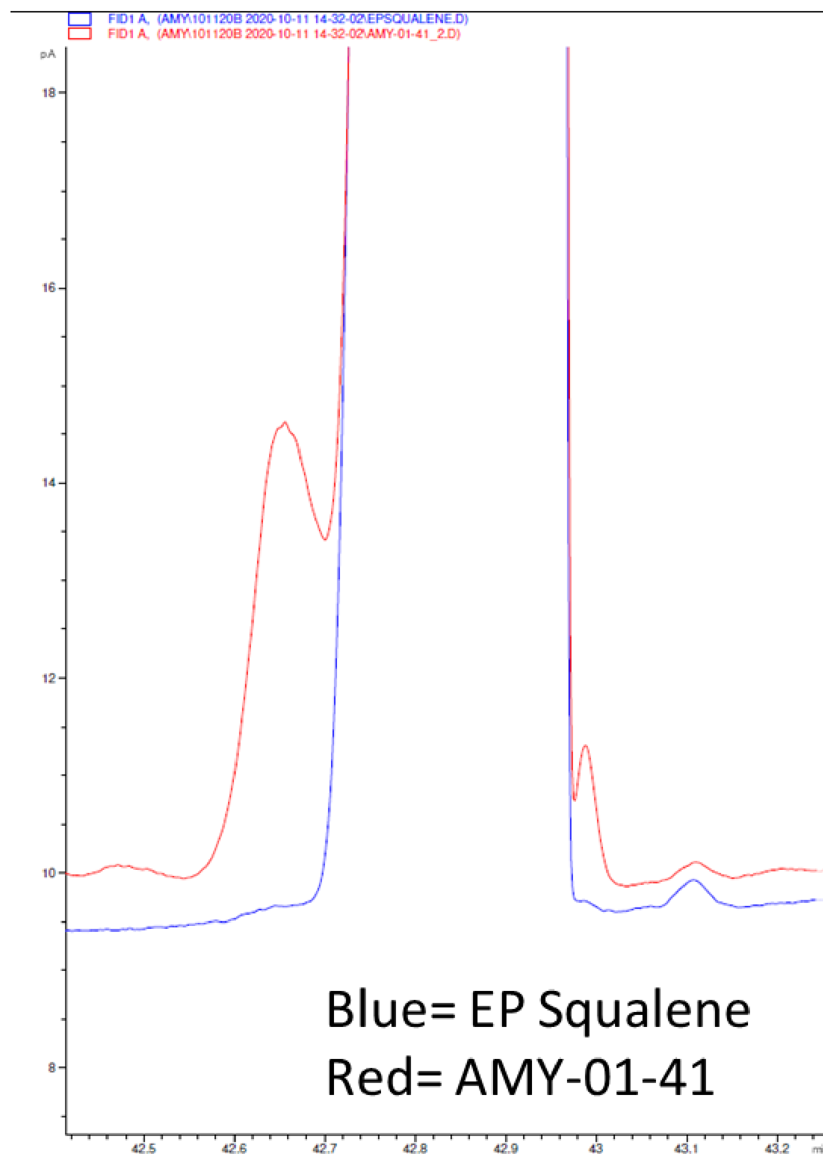


Figure S2: High-resolution gas chromatography separation of EDQM reference squalene standard (blue trace) and a sample of semi-synthetic squalene (red trace).

With this change the determined purity of the squalene peak in the example was reduced from 98.0% to 97.2%, confirming our suspicions that the purity was being overestimated. Based on the chemistry used and MS data (these peaks share the same *m/z* 410 value as squalene) it is assumed that these minor peaks correspond to small trace amounts of squalene isomers formed in the desulfonylation step. [3]

The second issue was the new impurities inherent to the chemistry used, in particular several peaks observed consistently eluting after the squalene peak (Figure S3).

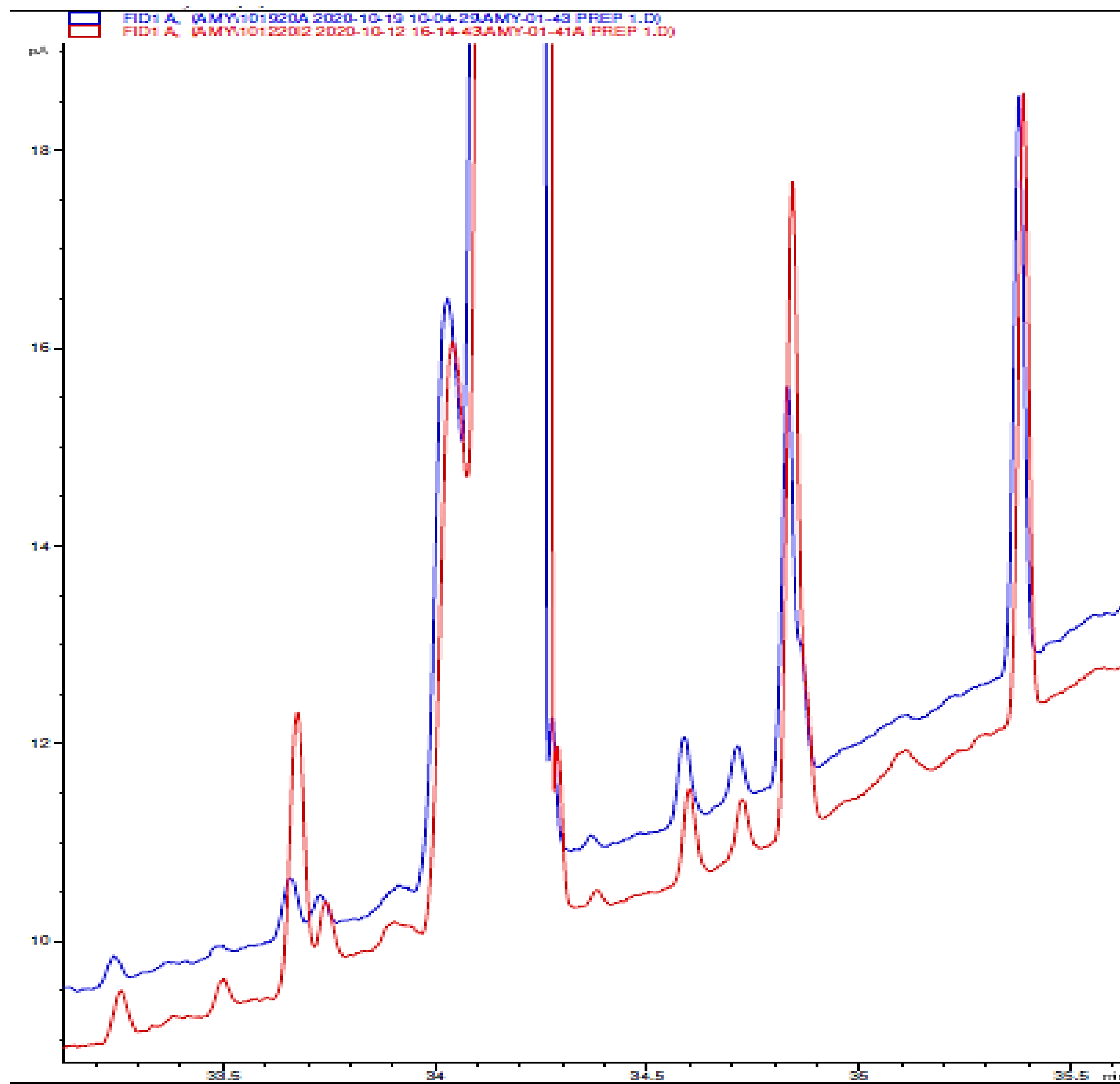
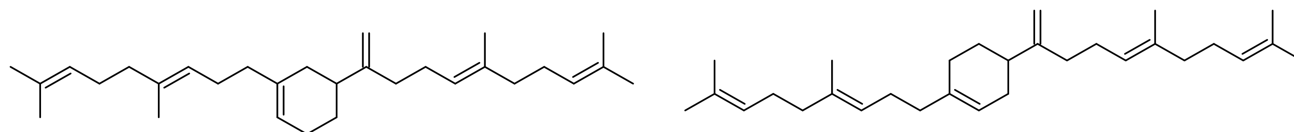


Figure S3: High resolution gas chromatograms of two different developmental lots of semi-synthetic squalene

In this case, based on the MS data (*m/z* 408 vs. 410 for squalene), our knowledge of the characteristic reactions of *trans*-β-farnesene, and the particular pattern of the peaks, it was

suspected these corresponded to the known farnesene thermal Diels Alder dimers.[4] This was confirmed by co-injection with an authentic sample of dimer mixture prepared from *trans*-β-farnesene. [4] The structures of these impurities are shown in Figure S4.

Major isomers



Minor isomers

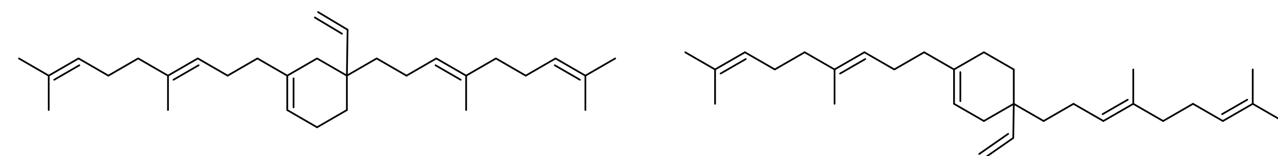


Figure S4: Structures of the thermal Diels Alder adducts of trans-β-farnesene

Additionally, an HPLC-DAD method (HPLC-IPC) was developed to monitor the synthesis and purity of the two sulfone-containing intermediates, which due to their lack of volatility and/or instability, could not be analyzed via the either the EP or the developed GC-IPC methods.

Details of both developed methods are given in the Experimental section of this SI.

Chemistry

Syntheses of farnesyl chloride (RSM 1) and farnesyl tolyl sulfone (RSM 2)

The two-step formation of RSM 1 proved uneventful. [4] We were already confident of the scalability of the first reaction, the formation of diethylfarnesylamine from farnesene, as identical chemistry, albeit using as substrate myrcene, the C_{10} analog of *trans*-β-farnesene, is already used industrially in the Takasago process for the manufacture of synthetic menthol on several thousands of tons/year scale. [5] An extended preliminary 100 g scale farnesene to diethyl

farnesylamine reaction using the optimum ratio (as determined by screening experiments) of 1.5 equivalents of diethylamine, and 0.08 equivalents of *n*-butyllithium, was performed to evaluate the reaction rate at 50°C and monitor any potential buildup of reagents. The results are shown in Table S1 and plotted in Figure S5. Based on these results, no buildup of reagents during a 5-6 hour farnesene addition at 50-60°C should be expected.

Time (h)	Farnesene %	Amine %
0	100	0
1	47.0	53.0
3	23.8	76.2
4	13.3	86.7
5	7.39	92.6
Hold at 19 to 14°C over 15 h	4.39	95.6

Table S1: Formation of diethylfarnesylamine from farnesene at 50°C. See text for details.

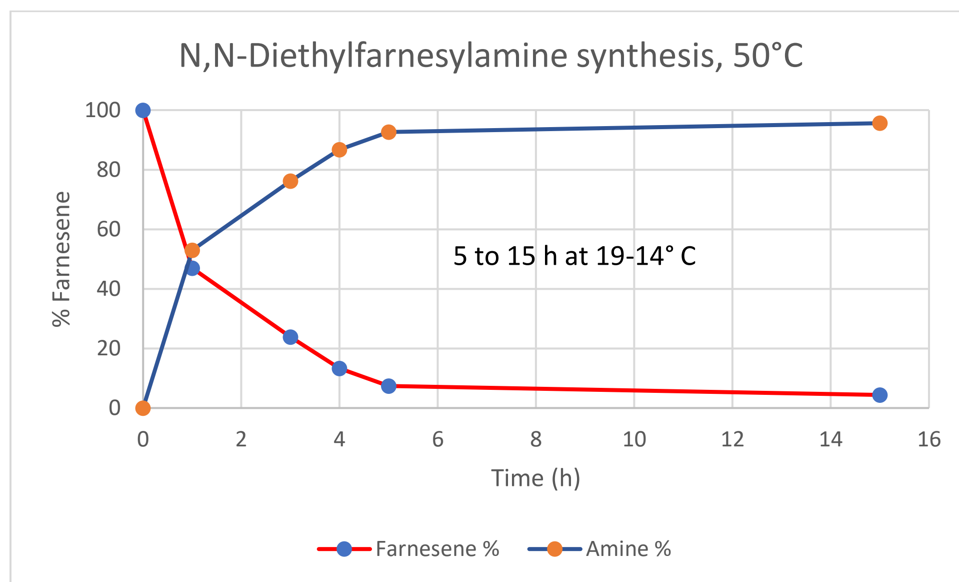


Figure S5. Formation of N.N-diethylfarnesylamine from farnesene at 50°C. Plot of data in Table S1. See text for details.

The main difference from our previous work [4] was that the N,N-diethylfarnesylamine product was subjected to purification as the corresponding acetate salt before proceeding to the next step

to remove unreacted farnesene and small amounts of the known farnesene Diels-Alder dimers [4], that carry through the subsequent reaction sequence and are not sufficiently removed in the final squalene purification.

Next a tertiary amine to chloride conversion using ethyl chloroformate in dichloromethane [6] provided the desired RSM 1 in good yield. As might be expected, this product is unstable and readily eliminates HCl to revert to farnesene, so simply drying to constant weight under mild conditions (no distillation) and cold storage between synthesis and use are recommended.

Formation of farnesyltolylsulfone (RSM 2) was readily accomplished by treating RSM 1 with sodium *p*-toluenesulfinate. Whereas in our original work [6] we used sodium phenylsulfinate, in this work it was found that the use of more economical sodium *p*-toluenesulfinate offered the additional advantage of providing a low melting crystalline sulfone, as opposed to the oil produced when the phenyl sulfone was prepared, thus facilitating purification.

The literature on sulfone synthesis[7] frequently mentions the use of additives such as tetrabutylammonium bromide and our original route[6] also used this reagent. Screening of alternate salts and additive levels revealed that the best result was obtained using 0.1 equivalents of tetrabutylammonium iodide, as reflected by a reproducible reduction of the levels of a late eluting impurity from 7% to 4%.

EXPERIMENTAL SECTION

General Information

Chemistry

Reagents and solvents were acquired from commercial suppliers and used as received, without further purification, unless otherwise noted. Squalene reference material (Ph. Eur. Squalene CRS reference material, catalog number Y0002131, “as is” $C_{30}H_{50}$ content 99.8%) was purchased from the EQDM Webstore (https://store.edqm.eu/index.html). *trans*-β-farnesene produced by yeast fermentation was supplied by Amyris. ^{1}H-and ^{13}C-NMR spectra were recorded on Bruker Advance spectrometers operating at frequencies of 300, 500 or 600 and 75, 125 or 150 MHz, respectively. IR spectra were recorded in the 500-4000 cm^{-1} range on a JASCO FT-IR/4600 spectrometer equipped with an ATR Pro One accessory.

Analytical Methods

The details of the developed GC method (GC-IPC) are given in Table S2.

<table>
<tr><th>Parameter</th><th colspan="3">Condition</th></tr>
<tr><td>GC System</td><td colspan="3">Agilent Series equipped with FID Detector, or equivalent</td></tr>
<tr><td>Column</td><td colspan="3">HP-1, 50 m x 200 µm x 0.11 µm, Agilent Part Number: 19091Z-005</td></tr>
<tr><td>Initial Oven Temperature</td><td colspan="3">90°C (1 min equilibration time)</td></tr>
<tr><td rowspan="3">Oven Program</td><td>Ramp (°C/min)</td><td>Final Temp (°C)</td><td>Final Time (min)</td></tr>
<tr><td>10.00</td><td>220</td><td>15.0</td></tr>
<tr><td>10.00</td><td>300</td><td>3.0</td></tr>
<tr><td>Run Time</td><td colspan="3">39.0 min</td></tr>
<tr><td>Inlet</td><td colspan="3">Split; 5:1</td></tr>
<tr><td>Inlet Temperature</td><td colspan="3">250°C</td></tr>
<tr><td>Gases</td><td colspan="3">Hydrogen: 45.0 mL/min
Air Flow: 450.0 mL/min
Combined Flow: 35.0 mL/min
Makeup Gas: Helium</td></tr>
<tr><td>Column Flow</td><td colspan="3">1.5 mL/min (constant flow)</td></tr>
<tr><td>FID Temperature</td><td colspan="3">300 °C</td></tr>
<tr><td>Injection Volume</td><td colspan="3">1 µL</td></tr>
</table>

Data Collection Rate	5 Hz
Injector Program	Three sample washes Four sample pumps Five post-injection acetone washes Five post-injection *n*-heptane washes

Table S2: GC-IPC procedure

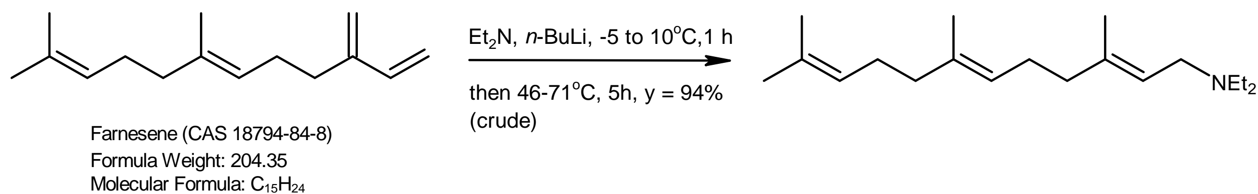
The details of the developed HPLC-DAD method (HPLC-IPC) are given in Table S3.

Parameter	Condition		
HPLC System	Agilent Series 1100 equipped with DAD Detector, or equivalent		
Column	Phenomenex Biphenyl, 100 x 3.0 mm, 2.6 µm, Part Number: 00D-4622-YO		
Column Temperature	40.0°C		
Autosampler Temperature	Ambient		
Mobile Phase A (MPA)	0.1% Phosphoric acid in water		
Mobile Phase B (MPB)	Acetonitrile		
Needle Wash	THF (wash 5 times pre-injection)		
Injection Volume	2 µL		
Run Time	15.0 minutes		
Detection Wavelength	210 nm		
Flow Rate	0.75 mL/min		
Diluent	Acetonitrile		
Gradient Profile	Time (Minutes)	% Mobile Phase A	% Mobile Phase B
	0.0	20	80
	5.0	20	80
	6.0	0	100
	8.0	0	100
	8.1	20	80
	15.0	20	80

Table S3: HPLC-IPC procedure

Chemistry

Step 1: Synthesis of crude *N,N*-diethylfarnesylamine from farnesene



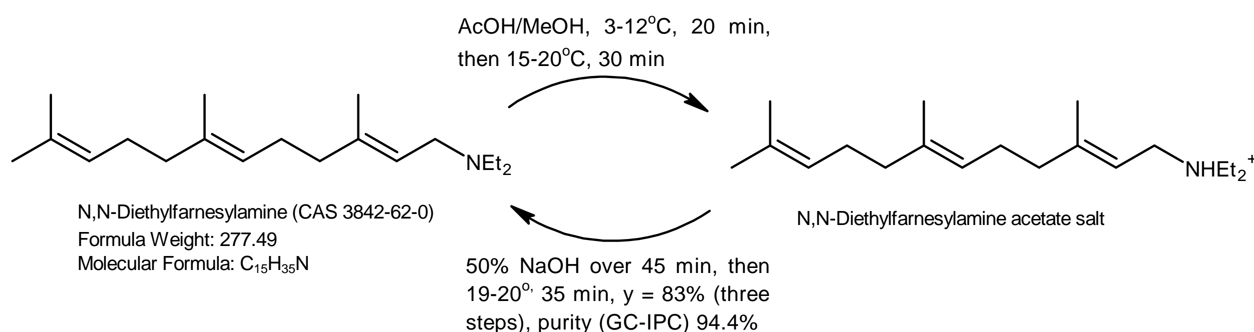
Scheme S1: Synthesis of N,N-diethylfarnesylamine

Diethylamine (DEA) (11.9 L, 8.40 kg, 115.0 mol, 1.5 eq) was charged to a nitrogen purged 100 L jacketed glass reactor fitted with an agitator, a cold-water condenser, and connected to a Huber chiller. The diethylamine was cooled to 0.8°C and *n*-butyllithium (1.70 kg, 2.45 L, 2.5 M solution in hexanes, 0.08 eq.) was added via a large bore cannula over 1.1 hours at −5.0 to +9.8°C. The DEA/lithium diethylamide reaction mixture was then heated to ~45°C and farnesene (15.60 kg, 19.2 liters, 76.4 mol) was added over 4.2 hours at 46-71°C via a ¼" double-diaphragm pump in 0.20 kg (near the beginning) to 0.55 kg portions (near the end). After the farnesene addition was finished, the reaction mixture was held at 67.5-71.2°C for 30 minutes, then cooled to 15°C with an increased nitrogen bleed to prevent air and/or oil from being pulled into the reactor as it cooled. After cooling the nitrogen bleed was reset. The slightly turbid and dark reaction mixture was held overnight (~14 h) under nitrogen at 15°C. A GC in-process check (GC-IPC) indicated 5.6% unreacted farnesene.

The dark reaction mixture turned a deep orange color as it was quenched with 5% potassium sodium tartrate (Rochelle's salt, 470 mL) at 12-25°C. The quench is exothermic during the first ~1/3 of the addition and an approximate 10-12°C temperature rise can be expected. The crude mixture was then vacuum stripped (35°C/~20 Torr, 4.5 L of volatiles removed). Heptane (15.8 L) and 5% Rochelle's salt (8.0 kg, 7.8 L) were added (51.5 L total volume) next and the two-phase mixture was stirred for 40 minutes, then allowed to settle for 1.5 hours. The dark-amber bottom aqueous layer (7.65 kg) was removed. The upper light-amber heptane/product layer (44.7 L) was washed with deionized (DI) water (6.85 kg, 30 min stir, 45 minute settle). The light-amber bottom aqueous layer (7.35 kg) was removed, and the upper light-amber heptane/product layer (44 L) was washed again with DI water, 6.90 kg, 40 min stir, overnight 16 hour settle).

The bottom aqueous layer (7.00 kg) was removed and the upper heptane/product layer was used directly in the Diels Alder dimer/farnesene removal process without removing heptane. An estimated 20 kg of crude amine was made in this process.

Step 1B: Purification of N,N-Diethylfarnesylamine to Remove Unreacted Farnesene and Diels Alder Adducts



Scheme S2. Farnesene dimer removal from farnesylamine

Additional heptane (5.70 kg/8.2 L) was added to the crude *N,N*,-diethylfarnesylamine in heptane (estimated 20 kg amine in ~10.80 kg/15.6 L of heptane) in a nitrogen purged 100 L jacketed glass reactor fitted with an agitator, condenser, and connected to a Huber chiller. Methanol (18.90 kg/23.9 L) and deionized water (3.60 kg) were added. The mixture was cooled to 2.7°C and glacial acetic acid (1.19 equivalents, 5.20 kg, 4.95 L) was slowly added at 2.7-12.2°C over 19 minutes to a pH of 5 (27°C was the highest temperature reached at this stage during prior condition screening experiments). The mixture was agitated for an additional 30 minutes at 15-20°C, then allowed to settle for 30 minutes. The layers were separated (volumes: bottom layer, 42 L, top layer, 34 L; total ~76 L). The top heptane/dimer layer was set aside for disposal later.

The bottom layer containing the N,N-diethyfarnesylamine acetate salt was extracted with heptane (11.1 kg/16 L), agitated for 30 minutes at 190 rpm, then allowed to settle for 30 minutes. The layers were separated: the top layer (containing dimer/farnesene impurities, ~16-20 L) was combined with the first top heptane/impurity layer and set aside. Note: the combined upper heptane extracts containing dimer, farnesene and other non-polar impurities, were temporarily saved, and discarded later. Due to the difficulty in determining the layer interface during the separation the bottom layer containing the diethylfarnesylamine acetate salt was extracted once more with 1 L of heptane as a precautionary measure to avoid yield losses.

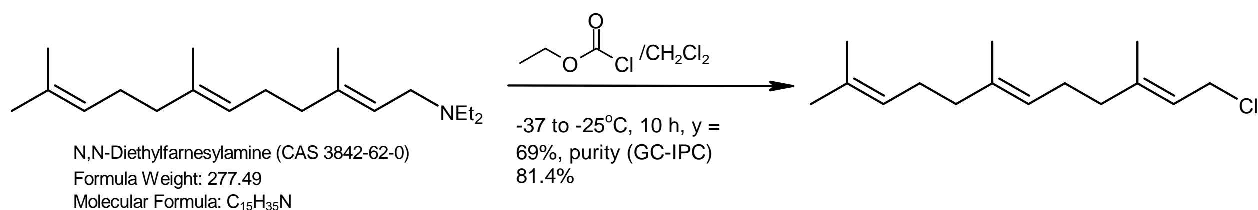
DI water (2.4 L) was added, the pH of the bottom layer was adjusted to 12 (using 0-14 pH paper) with 50% sodium hydroxide (1.19 equiv., 6.60 kg) at 14-20°C over 45 minutes with cooling at 0 ˚C. The biphasic mixture was agitated for 35 minutes at 19-20°C, and allowed to settle overnight (top layer: 30 L, bottom layer: 39 L). Note: the temperature was ~17°C and a substantial quantity of salts settled to the reactor bottom. DI water (3.0 L) was added and the mixture was briefly heated to 21°C to dissolve the salts. The layers were separated, the bottom layer was removed, and temporarily saved to be discarded later. The top heptane/product layer was washed with DI water (4.2 L) to remove residual sodium acetate (5 minute stir, 1 hour settle). Periodical jogging of the agitator was required to break up the emulsion formed.

The low-dimer *N,N*,-diethylfarnesylamine was concentrated to constant weight under reduced pressure (20-55°C, ~1 Torr). The amine was isolated as a light-yellow liquid (17.50 kg, 83% yield from farnesene). GC-IPC purity: overall 94.4%, 0.7% farnesene, 0.19% dimer.

1H-NMR (300 MHz, $CDCl_3$): 5.26 (t, 1H), 5.08 (q, 2H), 3.06 (d, 2H), 2.51 (q, 4H), 1.8-2.7 (m, 8H), 1.68 (bs, 3H), 1.64 (bs, 3H), 1.60 (bs, 3H), 1.03 (t, 6H).

$m/z = 277.3$

Step 2: Synthesis of Farnesyl Chloride (RSM 1) from *N,N*-Diethylfarnesylamine



Scheme S3. Synthesis of farnesyl chloride

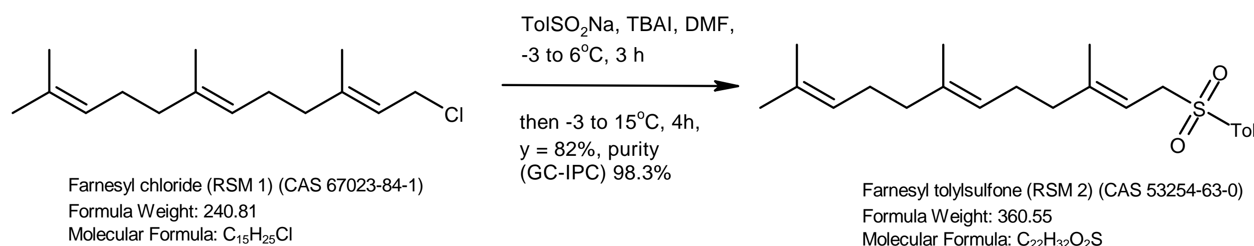
Dichloromethane (DCM) (60.05 kg) and ethyl chloroformate (7.53 kg, 6.60 L, 69.4 mol, 1.10 eq) were charged to a nitrogen purged 100 L jacketed glass reactor fitted with an agitator, a cold-water condenser, and connected to a Huber chiller. The solution was cooled to -36°C, and the remaining 3.55 kg of DCM was used to rinse empty ethyl chloroformate bottles into the reactor (63.60 kg total DCM was added). A solution of purified *N,N*,-diethylfarnesylamine (17.50 kg, 63.1 mol, 1.0 eq) and DCM (28.90 kg) was added over 7.9 hours at -37 to -25°C (keeping the 100L reactor temperature < -25°C). The reaction mixture was stirred at -27 to -31°C for 2.5 hours, then sampled, showing 1.7% starting amine (GC/MS IPC). An additional 50 mL of ethyl chloroformate was added to push the reaction to completion, and held overnight at -30°C. The reaction was quenched with a single portion of methanol (0.2 equivalents, 550 mL) added at -30°C. No exotherm was observed during the quench. DCM was removed by concentration under reduced pressure (to 0.8 Torr, 10-30°C water bath) to a constant weight (23.80 kg). The concentrated farnesyl chloride was then diluted with heptane (15 kg, 21.6 L), and washed sequentially with the following DI water/methanol solutions: 1 x (5.11 kg DI water/9.52 kg methanol) (30% v/v), 2 x (1.71 kg DI water/12.25 kg methanol) (10% v/v) and 2 x (0.86 kg DI

water/12.91 kg methanol) (5% v/v). Agitation/settling times were 15-45 minutes for each wash. The upper heptane/product solution was concentrated under reduced pressure (15-55°C/0.8 Torr) to constant weight. The chloride was isolated as a clear liquid (10.50 kg, 69% yield). GC-IPC purity: overall 81.4%, farnesene 5.3%, and dimers <0.1%.

^{1}H-NMR (300 MHz, $CDCl_3$): 5.45 (t, 1H), 5.09 (t, 2H), 4.10 (d, 2H), 2.05-2.15 (m, 6H), 1.93-2.03 (m, 2H), 1.73 (bs, 3H), 1.67 (bs, 3H), 1.60 (bs, 6H).

m/z = 240.2

Step 3: Synthesis of farnesyl tolyl sulfone (RSM 2) from farnesyl chloride (RSM 1) and sodium *p*-toluenesulfinate.



Scheme S4. Synthesis of farnesyl sulfone.

Farnesyl chloride (5.75 kg, 23.9 mol, 1.0 eq, 81.4% GC purity), DMF (5.45 kg, 5.75 L, 1.0 V), and TBAI (0.88 kg, 2.39 mol, 0.1 eq) were charged to a nitrogen purged 100 L jacketed glass reactor fitted with an agitator, condenser, and connected to a Huber chiller. The reaction mixture was cooled to -3.1 °C and sodium *p*-toluenesulfinate (4.45 kg, 24.99 mol, 1.047 eq) was added in 0.32-0.67 kg portions over 2.7 hours at -3.1-6.2°C (exothermic). Keeping the addition temperature below 15 °C should be sufficient for reaction success based upon small scale

screening experiments. The reaction was allowed to stir at -3.1-8°C for 1.5 hours and sampled. Stirring was continued until the IPC GC assay result (Table S4) indicated ≤1% unreacted RSM 1.

Time (h) (Total after finishing the addition)	% Farnesyl Chloride (GC)
0	NA
1.5	1.6
2.7	0.5

Table S4: In-process (IPC) test results.

At 3.6 hours after the addition, the reaction mixture was partitioned between heptane (7.75 kg, 11.2 L, 1.95 vol.) and DI water (7.60 kg, 1.33 vol., 2.5 – 14.8°C, chiller set at −10°C; the heptane should be added first to prevent the reaction mixture from solidifying). The turbid slurry was stirred for 40 minutes then filtered through #54 Whatman filter paper (18-inch HDPP Buchner funnel) to remove salts. As much of the heptane/aqueous mixture as possible was transferred back to the 100 L reactor, the filter salts were rinsed with heptane (1.6 L); and the rinse was transferred to a 6 L separatory funnel. The bottom aqueous layer in the separatory funnel was removed from the top heptane/product layer and added to the 100 L reactor. The remaining upper heptane/product layer (i.e., filter salts rinse) was saved for a later back-extraction of the aqueous layer. The 100 L layers were settled for 35 minutes, and the layers were separated and saved in separate containers. The bottom aqueous layer was added back to the 100 L reactor, and back-extracted with the heptane filter wash that was set aside earlier (30 minute stir, 20 minute settle). The bottom aqueous layer was removed. The upper heptane/product layers were combined in the 100 L reactor, and the line and pump were flushed with 1.6 L of heptane into the 100 L

reactor. The combined heptane/product layers were washed with 5% sodium thiosulfate (1 x 1.88 L, 27 min stir, 3 min settle) then with water (1 x 1.4 L, 25 min stir, 40 min settle), and finally with 37 (wt/wt)% (saturated) NaCl solution (1 x 1.80 L, 22 min stir, 1.2 h settle), Some emulsion was still present. The bottom aqueous layer was removed, and the emulsion layer was filtered through Whatman #54 paper and added to the 6 L separatory funnel. The bottom aqueous layer was removed, and the top heptane/product layer was added back to the 100 L reactor and cooled <u>with rapid agitation</u> (to prevent precipitation on the walls) while adding small aliquots of seed crystals (~ 10-20 mg per addition of material from a previous lot) in intervals as the solution was cooled, starting at 12.5°C. At around ~10.5-11 °C, the solution became turbid, and as crystals formed, the temperature increased from 9.6 to 11.7 °C at which point the solution became a stirrable slurry, which was then cooled to -4.8°C.

Before filtering, a cleaned 18-inch HDPP Buchner funnel was pre-cooled with dry ice, which was then removed prior to the next step. The slurry filtration started at -4.8 °C. A substantial quantity of solids remain in the reactor. To remove these about 10 L of the cold filtrate are added back to the 100 L reactor, and filtration is continued. Fairly rapid stirring is required to prevent the solids from settling, then the rpms were gradually decreased to allow the slurry to drain from the bottom. No wash on the wet cake is performed.

The wetcake is transferred to several crystallization dishes (8.20 kg total) and vacuum drying in a vacuum oven at ambient temperature was attempted. At this point, the wetcake had mostly melted to an oily slush in the 18—19°C room temperature vacuum oven and the sulfone still contained much heptane. The partially melted product was transferred to a 20 L rotary evaporator flask and concentrated under reduced pressure to 50-55°C/0.8 Torr to constant weight

(via freeze-thaw cycles to initially facilitate heptane removal). It was put through an additional freeze-thaw cycle then held at 50-55°C/0.8 Torr for 2.5 h. Yield: 7.14 kg (82%)

Purity (GC-IPC, air-dried wetcake sample): 98.3% farnesyl sulfone, 0.2% farnesene, 0.0% farnesyl chloride, ≤0.1% dimer.

Purity (GC-IPC, melt): 98.3% farnesyl sulfone, 0.3% farnesene, 0.0% farnesyl chloride, ≤0.1% dimer.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.79 – 7.73 (m, 2H), 7.36 – 7.31 (m, 2H), 5.20 (tq, *J* = 7.9, 1.3 Hz, 1H), 5.09 (ddddd, *J* = 13.9, 6.7, 5.4, 2.8, 1.5 Hz, 2H), 3.80 (d, *J* = 7.9 Hz, 2H), 2.46 (s, 3H), 2.12 – 1.99 (m, *J* = 9.3, 6.2 Hz, 8H), , 1.69 (q, *J* = 1.3 Hz, 3H), 1.61 (dd, *J* = 7.4, 1.4 Hz, 6H), 1.36 (d, *J* = 1.3 Hz, 3H).

^{13}C -NMR (126 MHz, $CDCl_3$): 146.21, 144.42, 135.88, 135.67, 131.38, 129.55, 128.56, 124.25, 123.39, 110.49, 56.16, 39.70, 26.70, 26.20, 25.70, 17.70, 16.25, 15.98

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