Shifting-Nitroxide to Investigate Enzymatic Hydrolysis of Fatty Acid by Lipases using Electron Paramagnetic Resonance in Turbid Media

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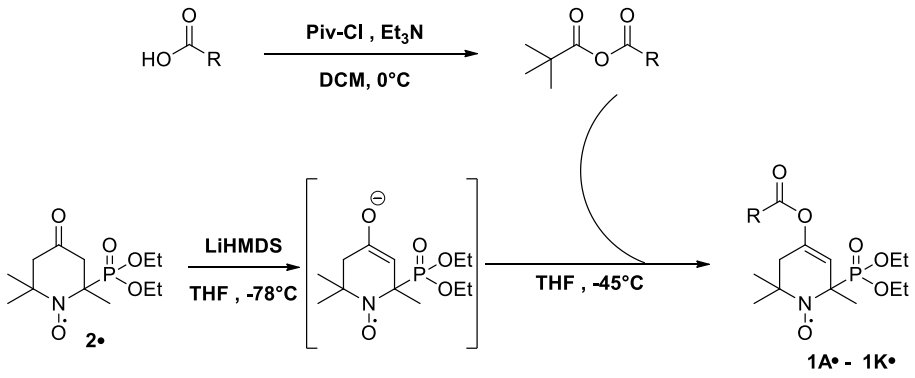
1. General comments

Fatty acids were purchased from TCI (linoleic and oleic acid) and from Supelco. Other chemicals were purchased from Sigma Aldrich and were used as received. All experiments were performed under anhydrous conditions and an inert atmosphere of argon and using dried apparatus and employing standard techniques for handling air-sensitive materials. Solvents were purchased from Aldrich and were dried using PureSolv micro solvent purification system. Solvents for purifications were used as technical grade. High-resolution mass spectra (HRMS) were performed on a SYNAPT G2 HDMS (Waters) spectrometer equipped with atmospheric pressure ionization source (API) pneumatically assisted. Samples were ionized by positive electrospray mode as follows: electrospray tension (ISV): 2800 V; opening tension (OR): 20 V; nebulization gas pressure (nitrogen): 800 L/h. The parent ion $[M + H]^+$ is quoted. Analytical thin layer chromatographies (TLC) were carried out on Merck Kieselgel 60 F254 plates. Flash column chromatographies were carried out on Merck Kieselgel 60 (230–400 mesh). For EPR measurements, samples were prepared in non-degassed solvents. Experiments were performed on EMX Bruker machines. EPR spectra were recorded with a gain of 25200, a modulation amplitude of 1.0 G, a sweep width of 100 G, a sweep time of 10 s, and a power of 20 mW.

2. Preparation of the Fatty acids – Nitroxide probes

Nitroxide **2•** was preparated as previously reported and the general procedure was adapted from a previously used strategy.[1]

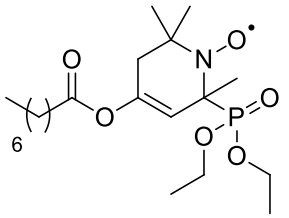
2.1. General procedure for the preparation Fatty acid – Nitroxide probes



To a solution of fatty acid (2 eq) in dry DCM (20 ml/mmol) at 0°C was added Pivaloyl chloride (3.4 eq) and triethylamine (2.6 eq) dropwise. The mixture was stirred for 1 hour at 0°C. DCM was then removed *in vacuo* and the residue obtained was taken up in diethyl ether. The suspension was filtered through a celite bed. Excess ether and pivaloyl chloride were removed under reduced pressure to yield the desired pivalic anhydride (quantitative) which was used in the next step without any further purification.

To a solution of nitroxide **2•** (1 eq) in dry THF at -78°C was added LiHMDS (1M in THF; 1.7 eq) and the mixture was stirred for 1.5 hours and was allowed to warm slowly at 0°C. The reaction mixture was then cooled to -45°C and a solution of freshly prepared pivalic anhydride (2 eq) in dry THF at -45°C was quickly added. The mixture was stirred for 2 to 4 hours (monitored by EPR and TLC) and was allowed to warm slowly at RT. On completion, the reaction was quenched by saturated aqueous solution ($NaCl/NH_4Cl$; 8/2; same volume as volume of THF used for reaction) and the aqueous layer was exctracted by EtOAc. The combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo. The resulting crude product was purified by column chromatography (Petroleum ether/Ethyl acetate; gradient from 90:10 to 50:50) to yield the desired probes as orange solids or oils.

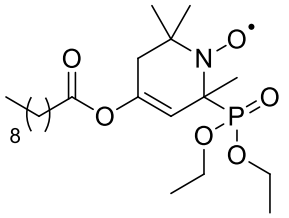
2.2. 2-(diethoxylphosphoryl)-4-heptylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1A•):



From caprylic acid (128 mg, 0.88 mmol), Et_3N (0.16 ml, 1.1 mmol), pivaloyl chloride (0.18 ml, 1.5 mmol), nitroxide **2•** (130 mg, 0.44 mmol), LiHMDS (1 M in THF, 0.75 ml, 0.75 mmol). **1A•** is obtained as an orange oil (108 mg, 59 %).

HRMS (ESI) calc for $C_{20}H_{38}NO_6P•^+$: 419.2431 $[M + H]^+$; found: 419.2431. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

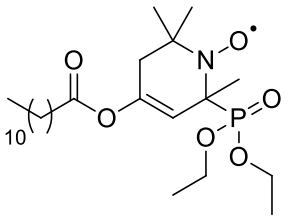
2.3. 2-(diethoxylphosphoryl)-4-nonylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1B•) :



From capric acid (118 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1B•** is obtained as an orange oil (91 mg, 60 %).

HRMS (ESI) calc for $C_{22}H_{42}NO_6P•^+$: 447.2744 $[M + H]^+$; found: 447.2744. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

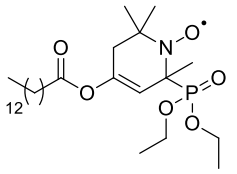
2.4. 2-(diethoxylphosphoryl)-4-undecylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1C•):



From lauric acid (137 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1C•** is obtained as an orange oil (93 mg, 57 %).

HRMS (ESI) calc for $C_{24}H_{46}NO_6P•^+$: 475.3057 $[M + H]^+$; found: 475.3058. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

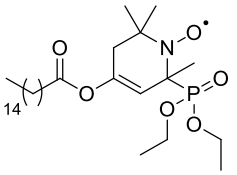
2.5. 2-(diethoxylphosphoryl)-4-tridecylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1D•):



From myristic acid (156 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol); pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1D•** is obtained as an orange oil (102 mg, 59 %).

HRMS (ESI) calc for $C_{26}H_{50}NO_6P•^+$: 503.3370 $[M + H]^+$; found: 503.3369. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

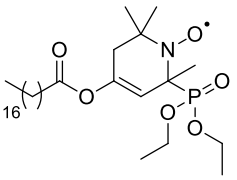
2.6. 2-(diethoxylphosphoryl)-4-pentadecylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1E•):



From palmitic acid (175 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1E•** obtained as an orange oil (118 mg, 65 %).

HRMS (ESI) calc for $C_{28}H_{54}NO_6P•^+$: 531.3683 $[M + H]^+$; found: 531.3685. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

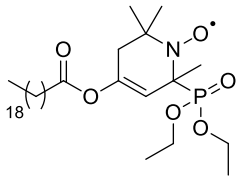
2.7. 2-(diethoxylphosphoryl)-4-heptadecylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1F•):



From stearic acid (195 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1F•** is obtained as an orange oil (120 mg, 63 %).

HRMS (ESI) calc for $C_{30}H_{58}NO_6P•^+$: 559.3996 $[M + H]^+$; found: 559.3995. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

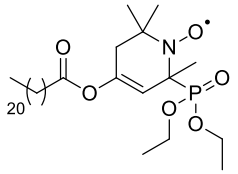
2.8. 2-(diethoxylphosphoryl)-4-nonadecylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1G•):



From arachidic acid (211 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1G•** is obtained as an orange solid (130 mg, 65 %).

HRMS (ESI) calc for $C_{32}H_{62}NO_6P•^+$: 587.4309 $[M + H]^+$; found: 587.4308. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

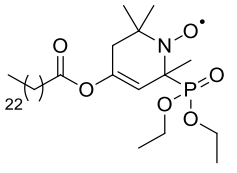
2.9. 2-(diethoxylphosphoryl)-4-heneicosylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1H•):



From behenic acid (234 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1H•** is obtained as an orange solid (127 mg, 61 %).

HRMS (ESI) calc for $C_{34}H_{66}NO_6P•^+$: 615.4622 $[M + H]^+$; found: 615.4623. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

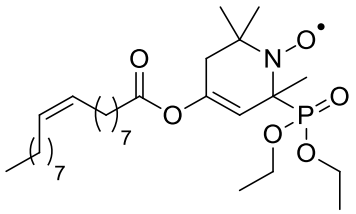
2.10. 2-(diethoxylphosphoryl)-4-tricosylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1I•):



From lignoceric acid (256 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1I•** is obtained as an orange solid (138 mg, 62 %).

HRMS (ESI) calc for $C_{36}H_{70}NO_6P•^+$: 643.4935 $[M + H]^+$; found: 643.4938. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

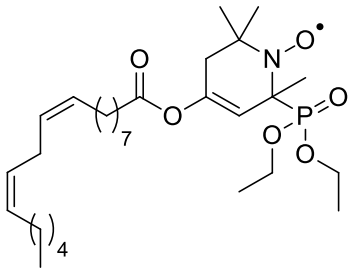
2.11. 2-(diethoxylphosphoryl)-4-heptadec-8-*cis*-enylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1J•):



From oleic acid (193 mg, 0.68 mmol), Et_3N (0.12 ml, 0.89 mmol), pivaloyl chloride (0.14 ml, 1.16 mmol), nitroxide **2•** (100 mg, 0.34 mmol), LiHMDS (1 M in THF, 0.58 ml, 0.58 mmol). **1J•** is obtained as an orange oil (123 mg, 64 %).

HRMS (ESI) calc for $C_{30}H_{56}NO_6P•^+$: 557.3840 $[M + H]^+$; found: 557.3840. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

2.12. 2-(diethoxylphosphoryl)-4-heptadec-8-*cis*,11-*cis*-dienylcarbonyloxy-2,6,6-trimethylpiperidin-3-en-N-oxyl radical (1K•):

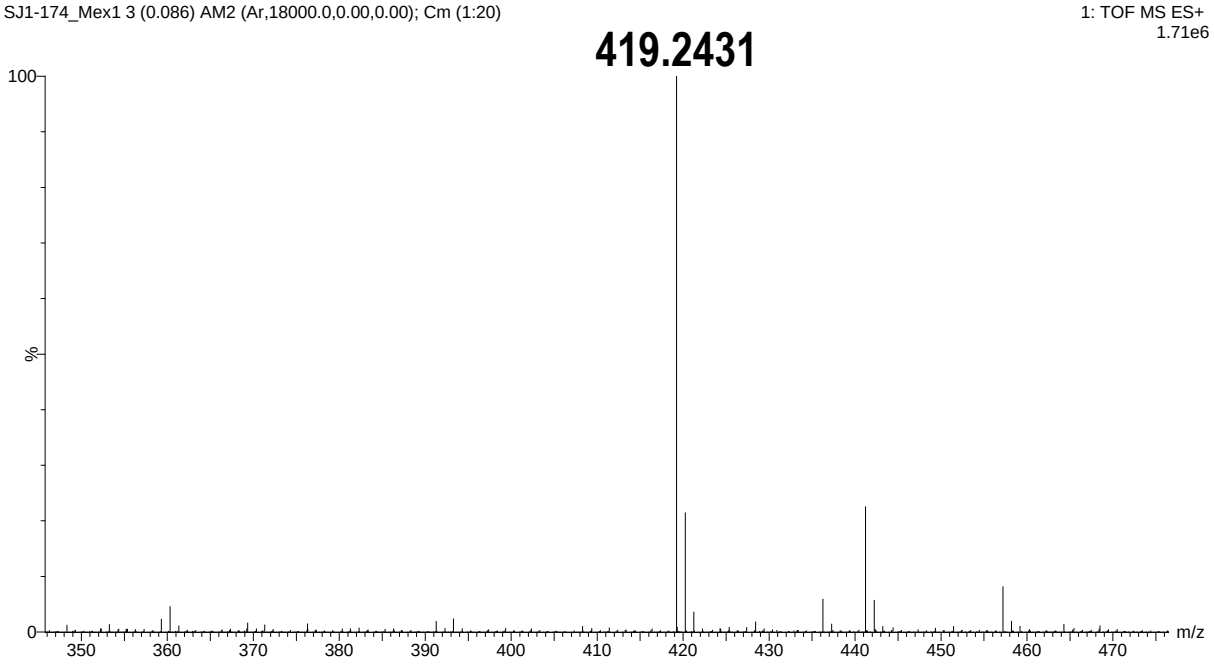


From linoleic acid (171 mg, 0.60 mmol), Et_3N (0.11 ml, 0.78 mmol:), pivaloyl chloride (0.13 ml, 1.02 mmol), nitroxide **2•** (90 mg, 0.30 mmol), LiHMDS (1 M in THF, 0.51 ml, 0.51 mmol). **1K•** is obtained as an orange oil (112 mg, 67 %).

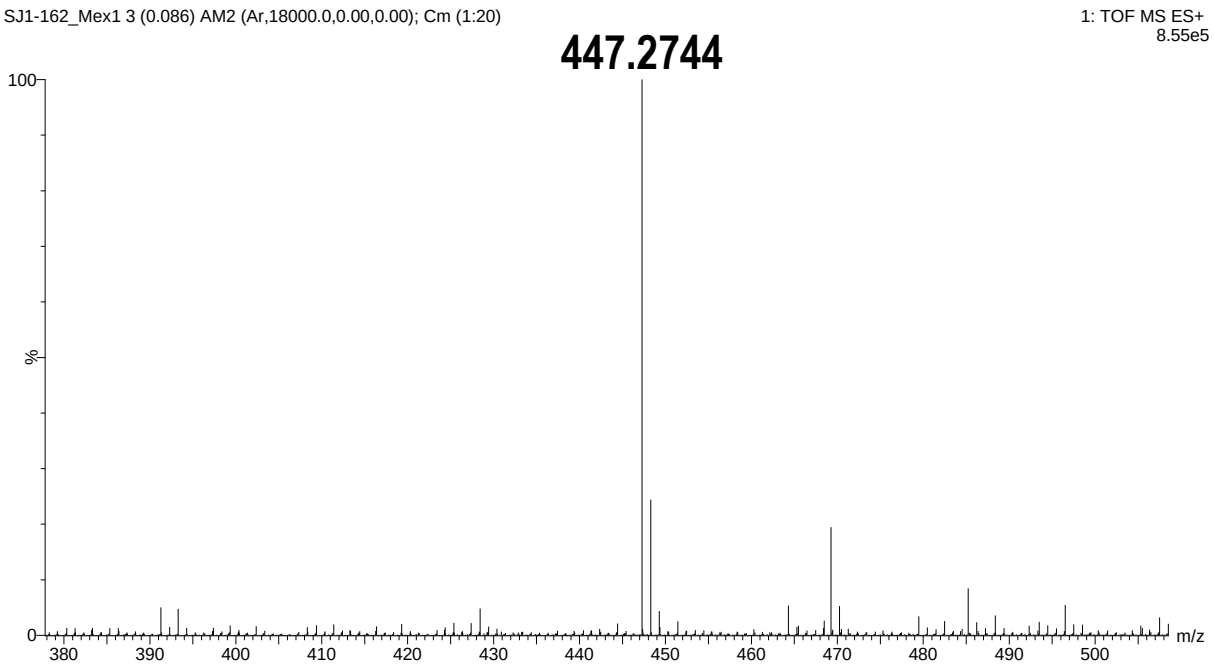
HRMS (ESI) calc for $C_{30}H_{56}NO_6P•^+$: 555.3683 $[M + H]^+$; found: 555.3681. EPR (CH_2Cl_2, 25°C): a_N: 14.6 G, a_P: 38.3 G.

3. HRMS spectra:

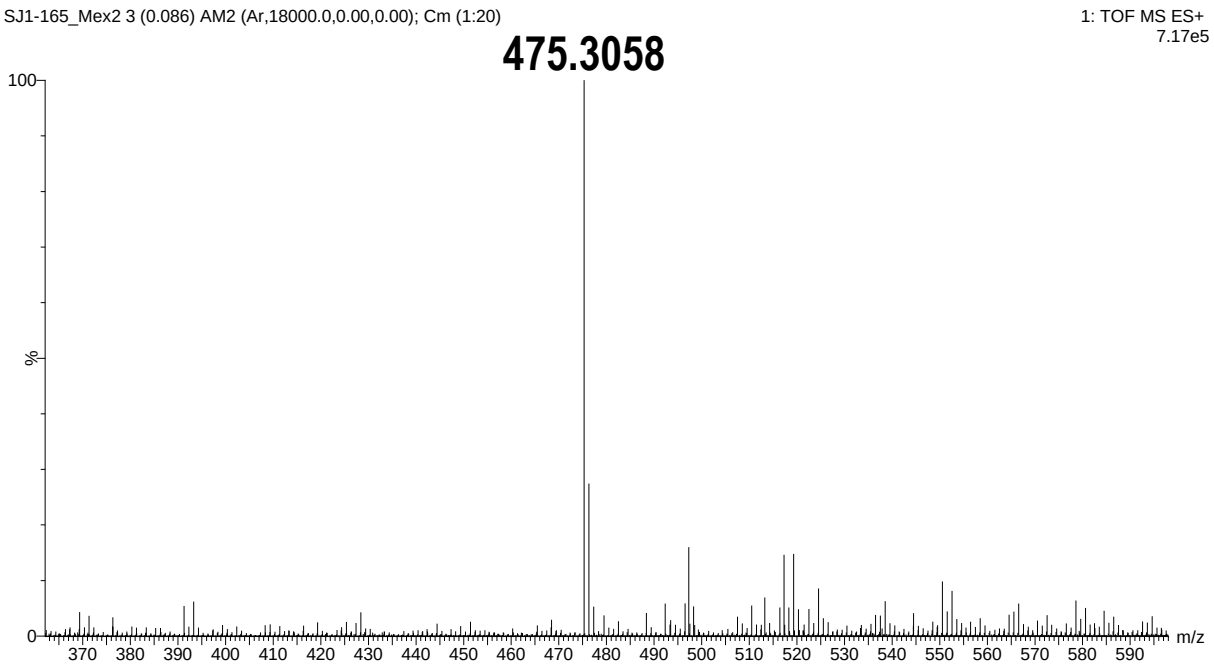
1A•



1B•



1C•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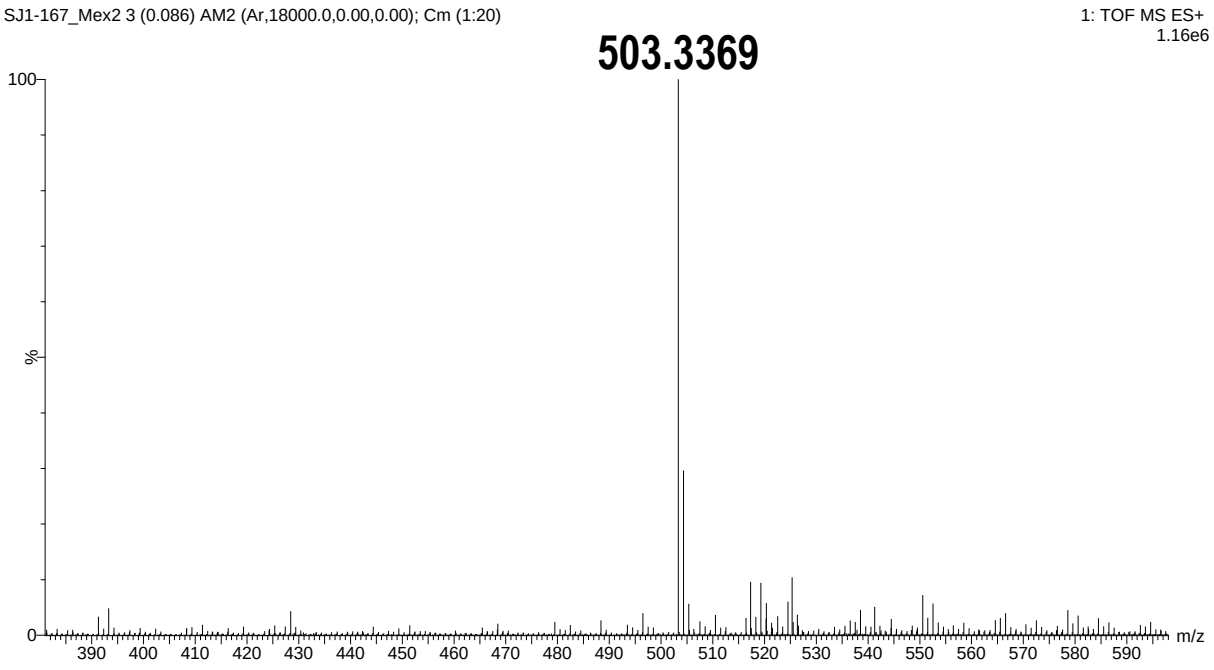
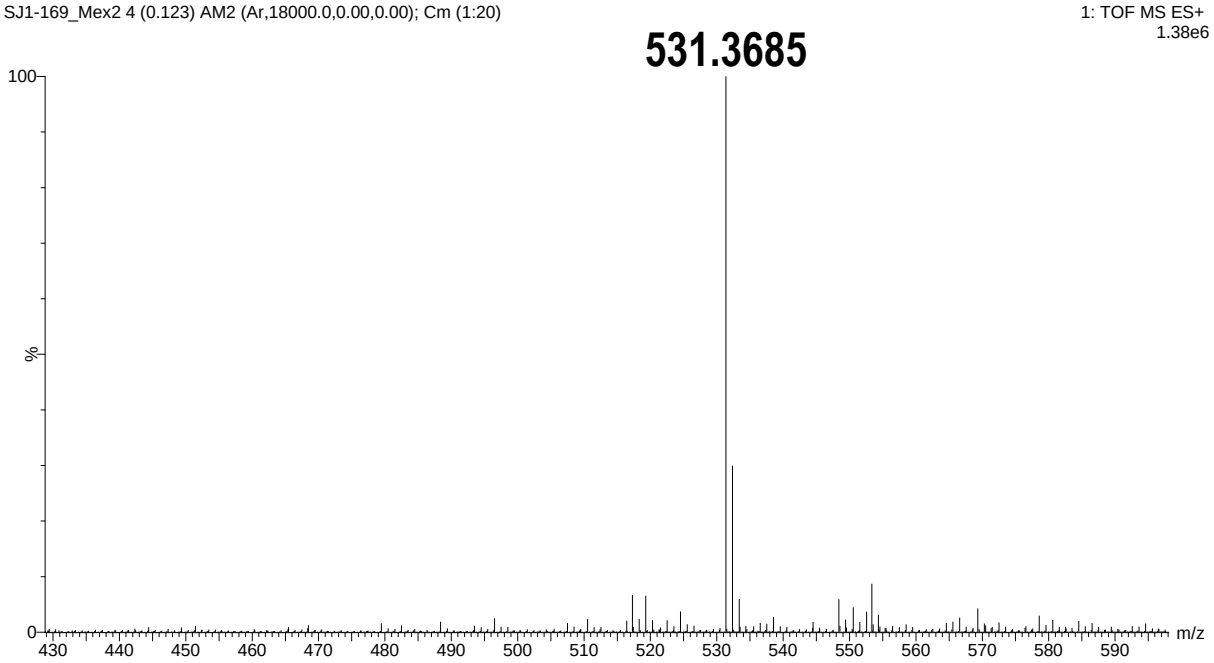
1: TOF MS ES+
7.17e5
475.3058
100
%
0
370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590
m/z

1D•


SJ1-167_Mex2 3 (0.086) AM2 (Ar,18000.0,0.00,0.00); Cm (1:20)
1: TOF MS ES+
1.16e6
503.3369
100
%
0
390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590
m/z

1E•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%
0
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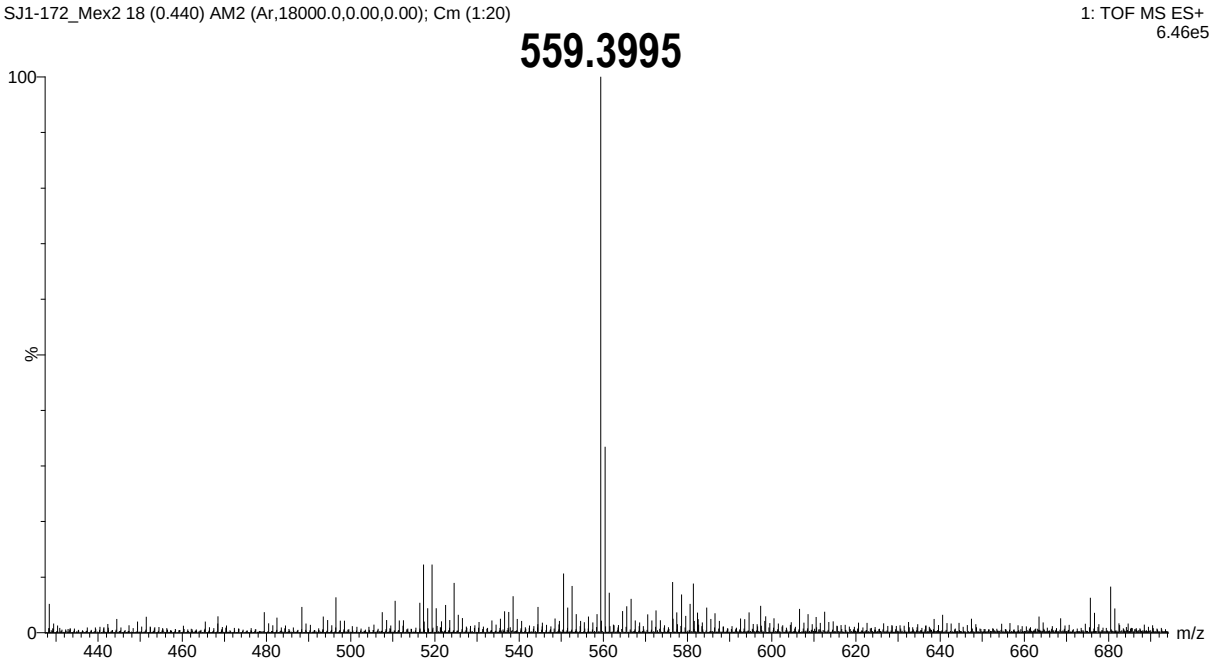
m/z

1F•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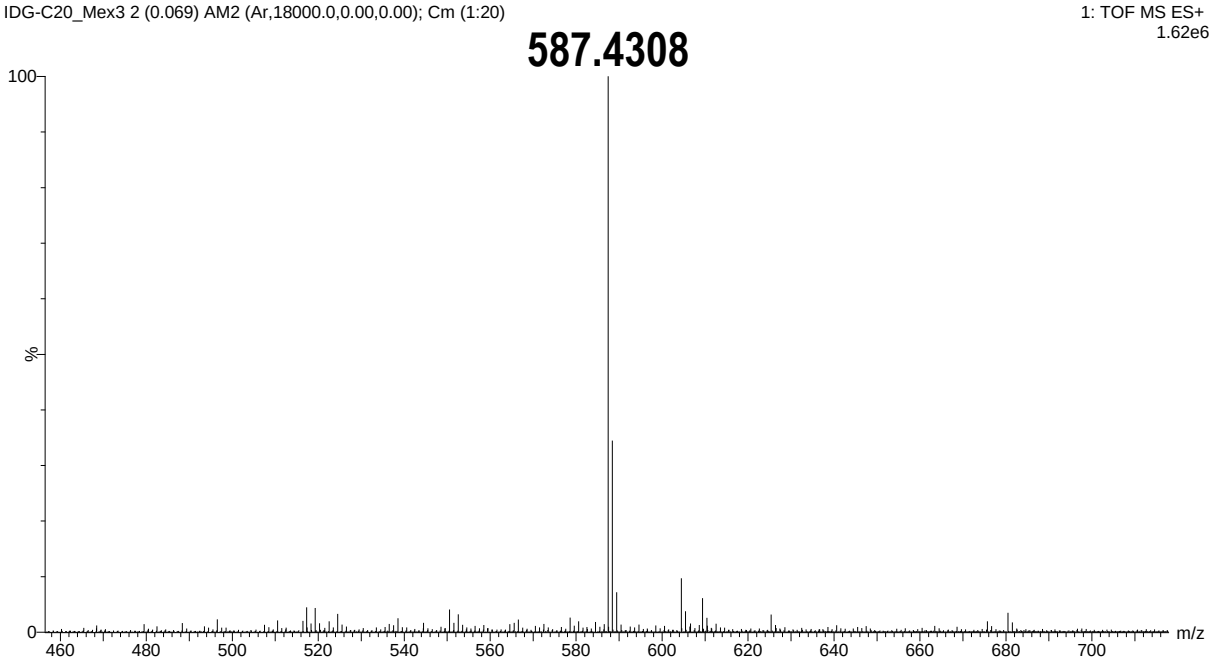
100
%
0
440 460 480 500 520 540 560 580 600 620 640 660 680
m/z

1G•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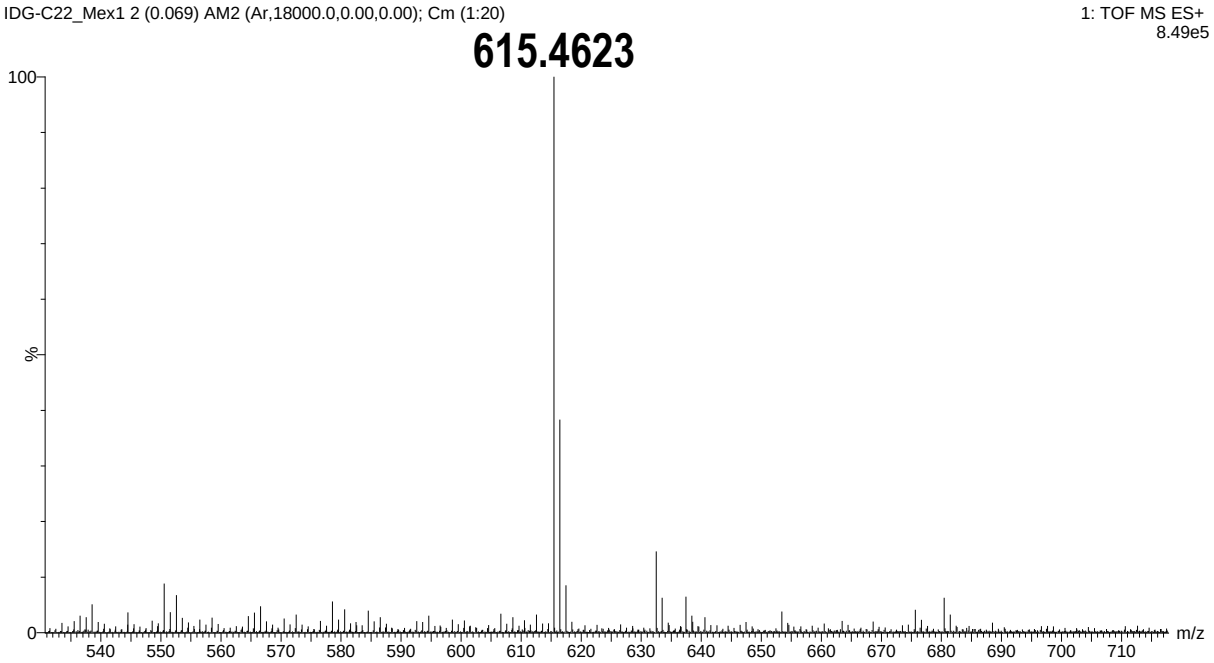
587.4308
100
%
0
460
480
500
520
540
560
580
600
620
640
660
680
700
m/z

1H•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615.4623
100
%
0
540
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560
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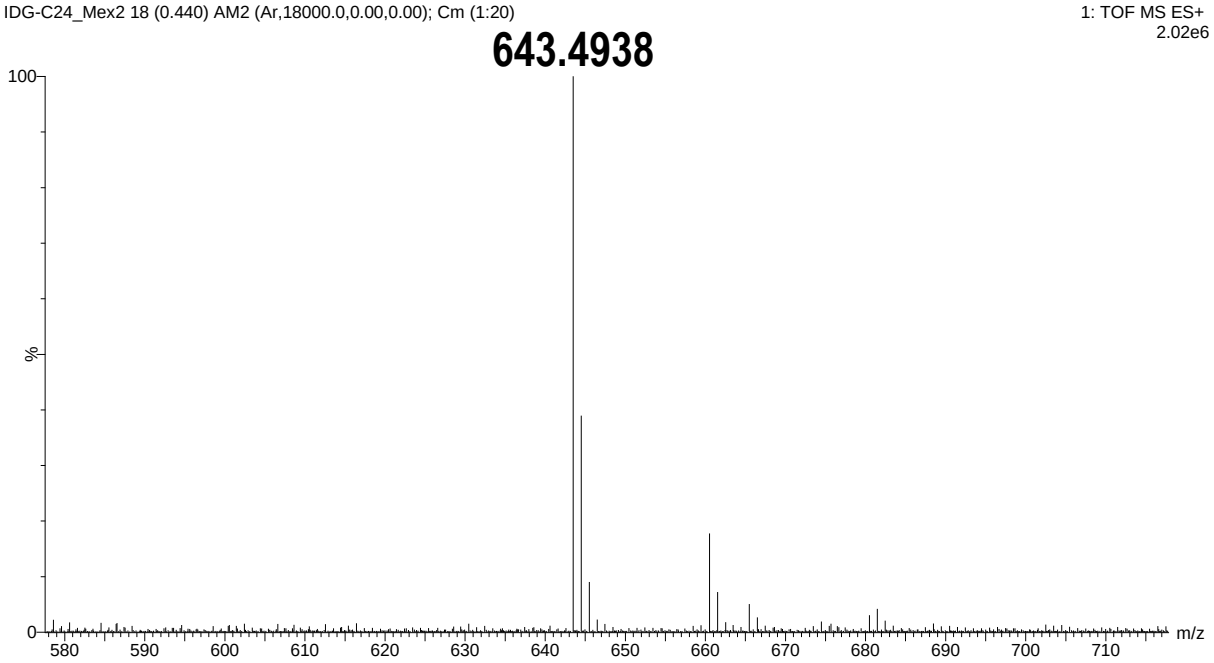
590
600
610
620
630
640
650
660
670
680
690
700
710
m/z

1I•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2.02e6
643.4938
100
%
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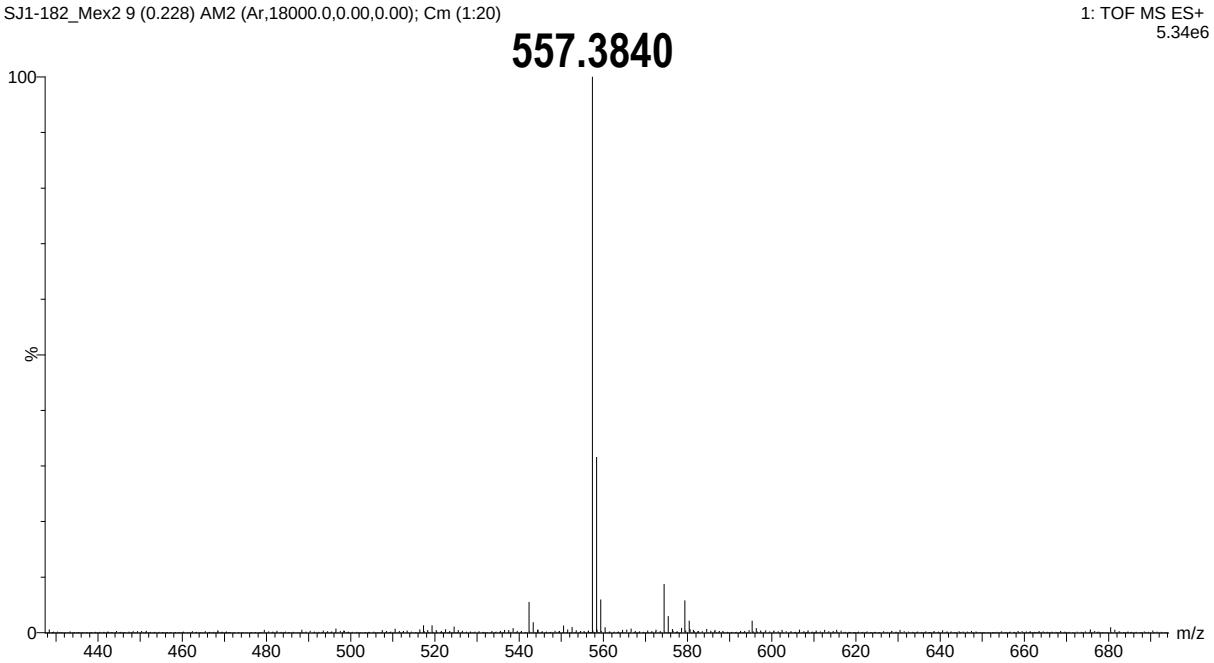
590
600
610
620
630
640
650
660
670
680
690
700
710
m/z

1J•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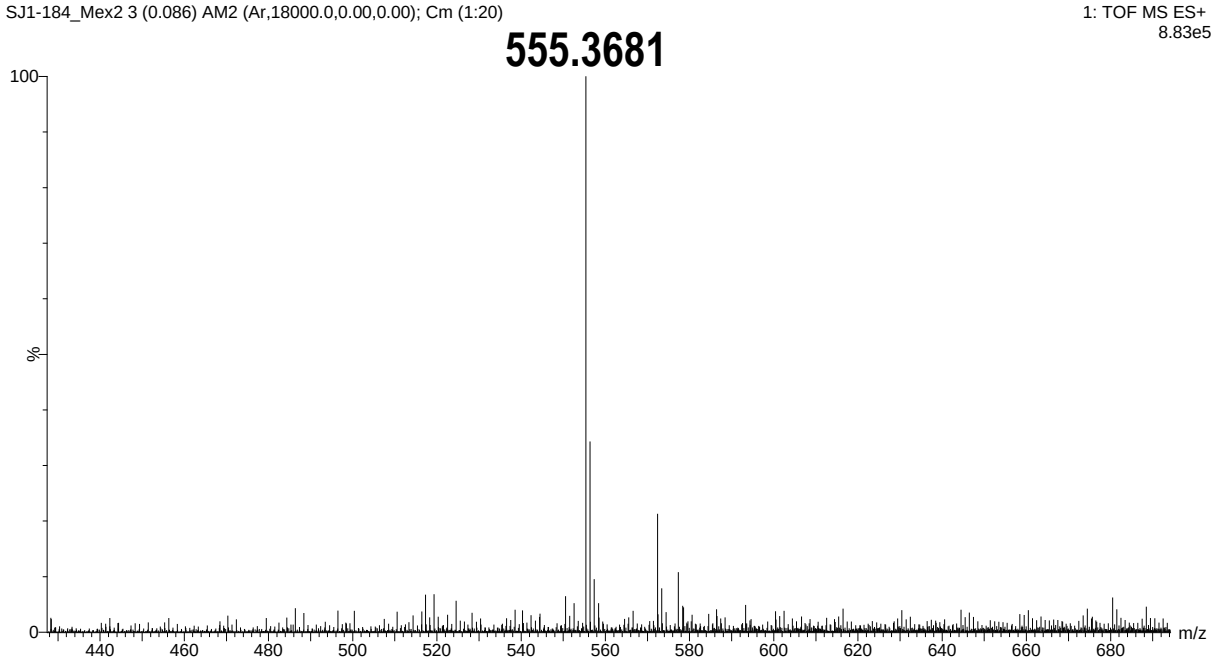
557.3840
100
%
0
440
460
480
500
520
540
560
580
600
620
640
660
680
m/z

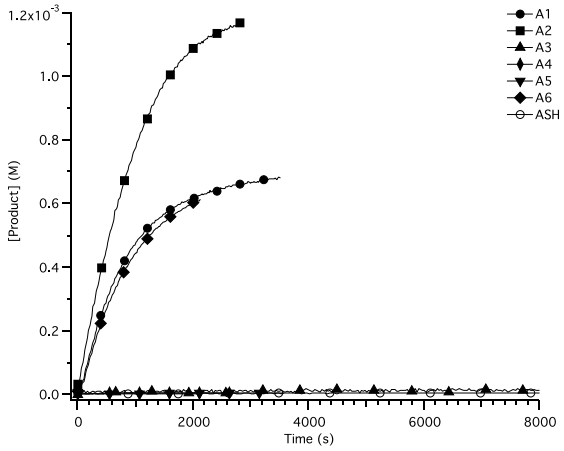
1K•



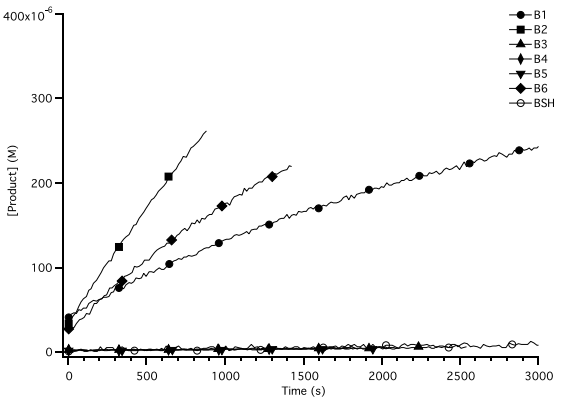
4. Kinetics

A list of 5 lipases, Porcine Pancreas type II (1) (Sigma-Aldrich, Germany), Candida rugosa (2) (Sigma-Aldrich, Germany), AK "Amano 20" (3) (Amano Pharmaceutical Co. Ltd., Nagoya, Japan), Candida antartica B (4) (Novo Nordisk, Denmark), and Mucor miehei (5) (Novo Nordisk, Denmark), was tested with various fatty acid - nitroxide substrates.

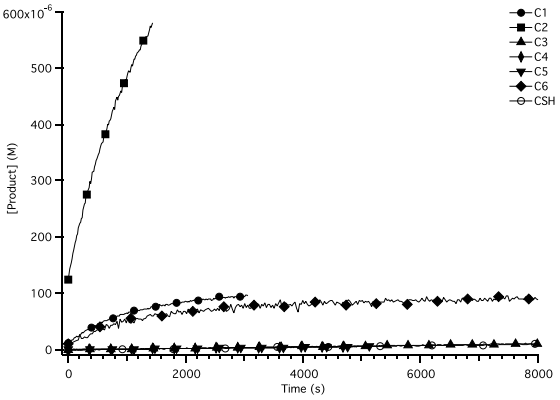
All experiments were performed with 1 mM of substrate. Enzymatic reactions were initiated by adding a small volume of lipase at 1 μM. Kinetic measurements were carried out at pH = 7,4 (buffer HEPES 50 mM, 0.15 M NaCl, IGEPAL 0.05%) and sample temperature was kept at 25°C or 40°C with a temperature controller (BIO-I, NOXYGEN, Germany). The spontaneous hydrolysis (SH) of substrates was evaluated by replacing the volume of lipase by the same volume of HEPES buffer. An EMXnano EPR spectrometer (BRUKER, Germany) was used for enzymatic reactions. Fatty acid - nitroxide probe samples were loaded in 60 μL / 75 mm capillaries (BLAUBRAND, Germany). Kinetics were immediately recorded by EPR spectrometer during 2-6 hours. EPR acquisition parameters were set as: B_0 = 3423 G, sweep width = 100 G, sweep time = 5 seconds, attenuation = 4-20 dB, modulation amplitude = 1 G, gain = 40 dB.


1.2x10-3
1.0
0.8
0.6
0.4
0.2
0.0
[Product] (M)
0
2000
4000
6000
8000
Time (s)
A1
A2
A3
A4
A5
A6
ASH

(A•)


400x10-6
300
200
100
0
[Product] (M)
0
500
1000
1500
2000
2500
3000
Time (s)
B1
B2
B3
B4
B5
B6
BSH

(B•)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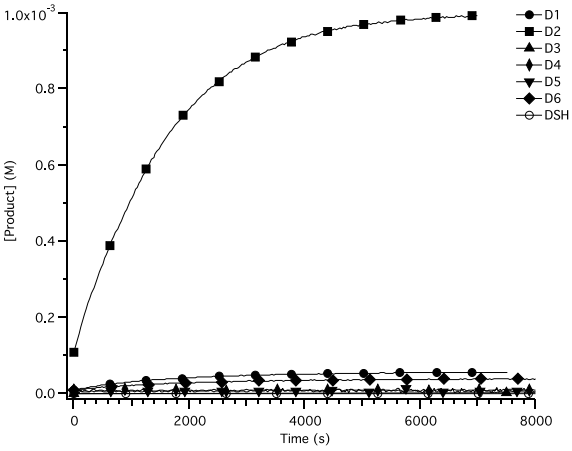
500
400
300
200
100
0
[Product] (M)
0
2000
4000
6000
8000
Time (s)
C1
C2
C3
C4
C5
C6
CSH

(C•)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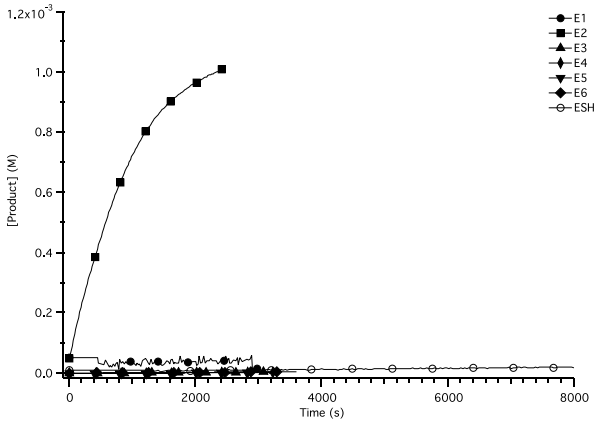
0.8
0.6
0.4
0.2
0.0
[Product] (M)
0
2000
4000
6000
8000
Time (s)
D1
D2
D3
D4
D5
D6
DSH

(D•)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0.2
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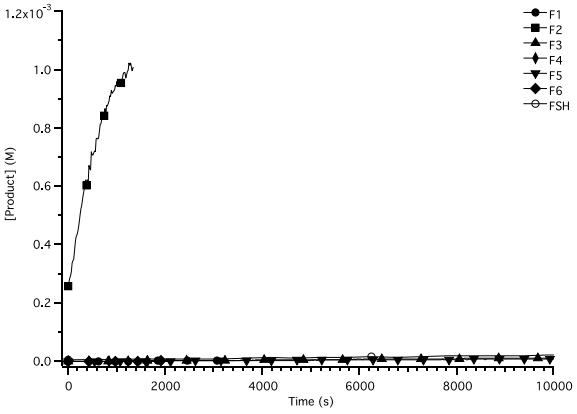
2000
4000
6000
8000
Time (s)
E1
E2
E3
E4
E5
E6
ESH

(E•)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0.8
0.6
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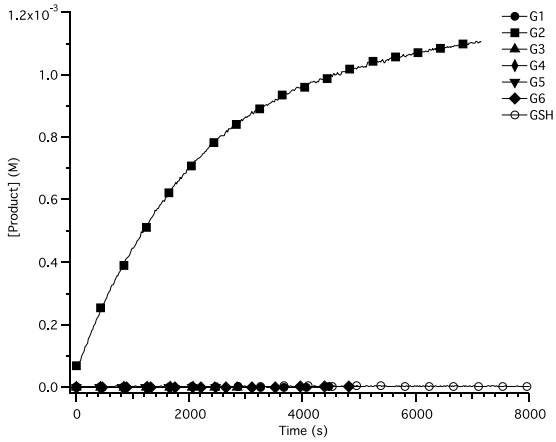
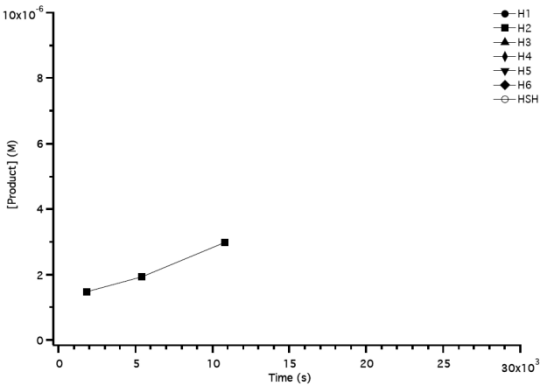
[Product] (M)
0
2000
4000
6000
8000
10000
Time (s)
F1
F2
F3
F4
F5
F6
FSH

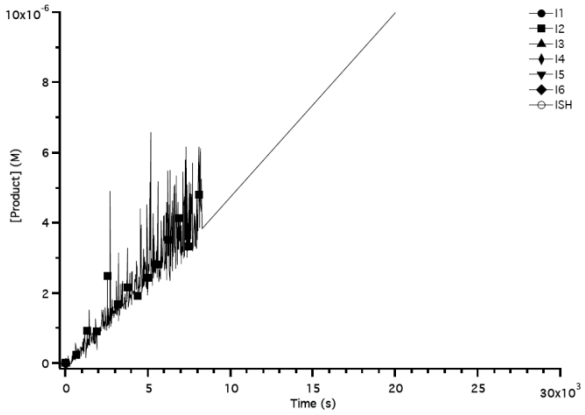
(F•)



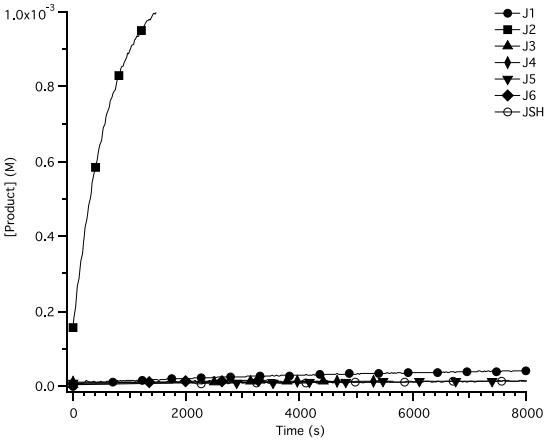
(**G•**)



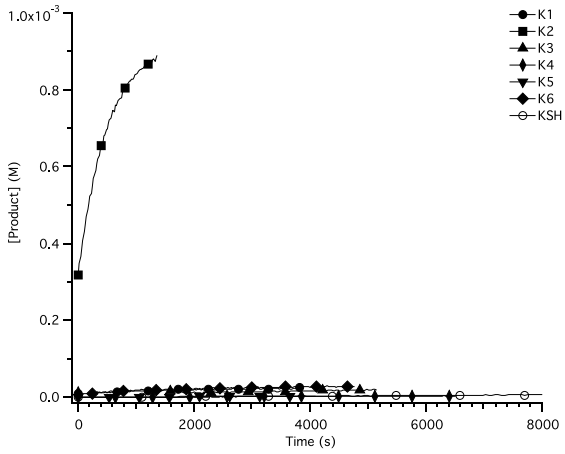
(**H•**)



(**I•**)



(**J•**)



(**K•**)

Figure 1SI. Curves of product generation kinetics from a range of lipases (1-6) at 1 μM and spontaneous hydrolysis (SH) of fatty acid - nitroxide substrates (**1A•-K•**) in HEPES buffer pH = 7.4 at 25 °C.

Initial velocities

Quantitation of product formation at each time of kinetics was done using the SpinCount calibrated module of the spectrometer. EPR spectra processed using IGOR Pro (Wavemetrics, Lake Oswego, OR, USA) enable quantification of product formation during the required time course. Initial velocities were measured for each curves (Figure.1SI) and compared according to each lipase/fatty acid - nitroxide couples (Figure.2SI, Table.1SI).

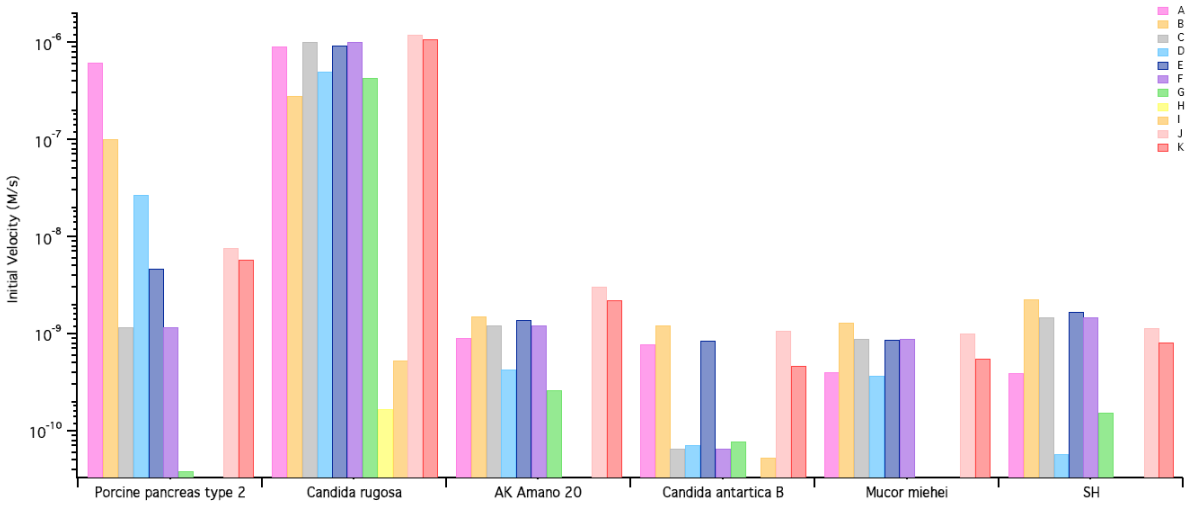


Figure 2SI. Comparison of initial velocities from kinetic with fatty acid - nitroxide substrates (**1A•-K•**) mixed to 1 µM of various lipases: Porcine pancreas type II, Candida rugose, AK Amano 20, Candida antartica B, and Mucor miehei. Spontaneous hydrolysis (SH) of each substrates in HEPES buffer are displayed as control. For better comparison, a logarithmic scale was used.

	initial velocity ($M^{-1}\cdot s^{-1}$)										
1•	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
PP type II	$6.2\ 10^{-7}$	$1.0\ 10^{-7}$	$1.1\ 10^{-9}$	$2.7\ 10^{-8}$	$4.6\ 10^{-9}$	$1.1\ 10^{-9}$	$3.8\ 10^{-11}$	n.d.	n.d.	$7.7\ 10^{-9}$	$5.8\ 10^{-9}$
Candida rugosa	$9.1\ 10^{-7}$	$2.8\ 10^{-7}$	$1.0\ 10^{-6}$	$5.0\ 10^{-7}$	$9.4\ 10^{-7}$	$1.0\ 10^{-6}$	$4.3\ 10^{-7}$	$1.7\ 10^{-10}$	$5.3\ 10^{-10}$	$1.1\ 10^{-6}$	$1.0\ 10^{-6}$
AK Amano 20	$9.1\ 10^{-10}$	$1.5\ 10^{-9}$	$1.2\ 10^{-9}$	$4.3\ 10^{-10}$	$1.3\ 10^{-9}$	$1.2\ 10^{-9}$	$2.6\ 10^{-10}$	n.d;	n.d.	$3.1\ 10^{-9}$	$2.2\ 10^{-9}$
Candida antartica B	$7.7\ 10^{-10}$	$1.2\ 10^{-9}$	$6.5\ 10^{-11}$	7.1e-11	$8.6\ 10^{-10}$	$6.5\ 10^{-11}$	$7.7\ 10^{-11}$	n.d.	n.d.	$1.0\ 10^{-9}$	$4.7\ 10^{-10}$
Mucor miehei	$4.1\ 10^{-10}$	$1.3\ 10^{-9}$	$8.9\ 10^{-10}$	$3.7\ 10^{-10}$	$8.7\ 10^{-10}$	$8.9\ 10^{-10}$	$3.3\ 10^{-11}$	n.d.	n.d.	$1.0\ 10^{-9}$	$5.6\ 10^{-10}$
SH	$5.6\ 10^{-7}$	$1.6\ 10^{-7}$	$1.2\ 10^{-10}$	$1.5\ 10^{-8}$	$1.2\ 10^{-9}$	$1.2\ 10^{-10}$	$2.4\ 10^{-10}$	n.d	n.d.	$2.6\ 10^{-9}$	$1.0\ 10^{-8}$

Table 1SI. Initial velocity values of 6 lipases against 11 different substrates named from A to K (n.d : non-detected, SH spontaneous hydrolysis).

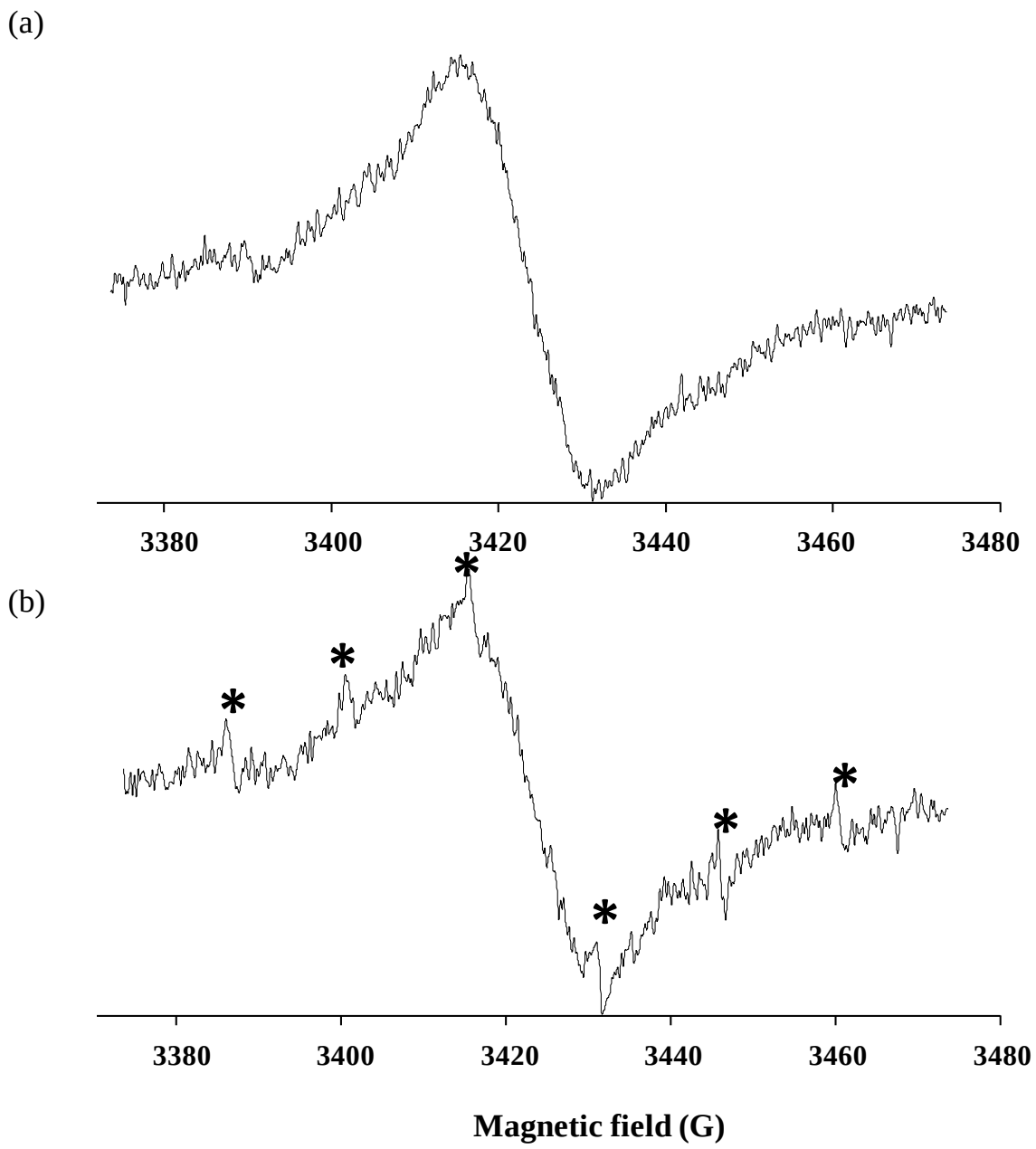


Figure 3SI. The particular case of substrate **1I•** at t = 0 (a) and at t = 4200s (b) at 25°C in the presence de 50 µg/ml of *Candida rugosa* lipase showing the product **2•** (stars, red star for the line used in kinetics) appearing superimposed on the broad line of the substrate.

5. References

[1] Duttagupta, I.; Jugniot, N.; Audran, G.; Franconi, J. M.; Marque, S. R. A.; Massot, P.; Mellet, P.; Parzy, E.; Thiaudière, E. ; Vanthuyne N. *Chem.Eur.J.* 2018, 24, 7615 –7619.