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Rat Hormone Sensitive Lipase Inhibition by Cyclipostins and Their Analogs

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Abstract

Cyclipostins are bicyclic lipophilic phosphate natural products. We report here that synthesized individual diastereomers of cyclipostins P and R have nanomolar IC_{50}s toward hormone sensitive lipase (HSL). The less potent diastereomers of these compounds have 10-fold weaker IC_{50}s. The monocyclic phosphate analog of cyclipostin P is nearly as potent as the bicyclic natural product. Bicyclic phosphonate analogs of both cyclipostins exhibit IC_{50}s similar to those of the weaker diastereomer phosphates (about 400 nM). The monocyclic phosphonate analog of cyclipostin P has similar potency. A series of monocyclic phosphonate analogs in which a hydrophobic tail extends from the lactone side of the ring are considerably poorer inhibitors, with IC_{50}s around 50 μM. Finally cyclophostin, a related natural product inhibitor of acetylcholinesterase (AChE) that lacks the hydrocarbon tail of cyclipostins, is not active against HSL. These results indicate a critical SAR for these compounds, the hydrophobic tail. The smaller lactone ring is not critical to activity, a similarity shared with cyclophostin and AChE. The HSL kinetics of inhibition for the cyclipostin P *trans* diastereomer were examined in detail. The reaction is irreversible with a K_I of 40 nM and a rate constant for inactivation of 0.2 min^{-1}. These results are similar to those observed for cyclophostin and AChE.

1. INTRODUCTION

The serine hydrolases constitute one of the largest classes of enzymes in biochemistry [1]. These biological catalysts share the classic α/β hydrolase fold structure and the well known catalytic triad composed of an acidic residue, His, and Ser. This last residue serves as the nucleophile in the attack on ester or amide substrates [2]. Among the serine hydrolases of therapeutic interest are acetylcholinesterase (AChE[1]), butyrylcholinesterase, and a number of lipases. For this reason, extensive literature has evolved from inhibition studies [3–5].

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Supplementary Material. Supplementary data associated with this article can be found, in the online version.

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[1]AChE, acetylcholinesterase; HSL, hormone sensitive lipase.

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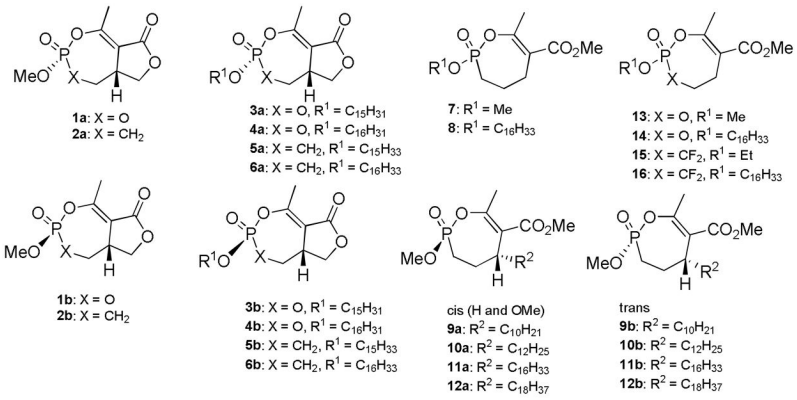
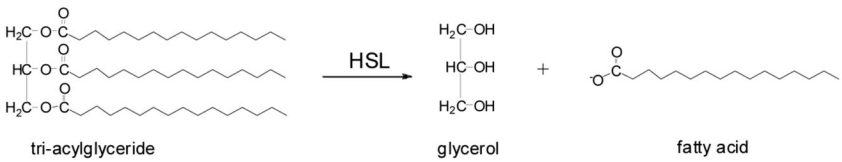
An interesting class of esterase inhibitors has emerged from the natural product literature. The basic structure is represented by cyclophostin **1a** (Scheme 1), a bicyclic phosphate isolated from *Streptomyces* species strain DSM 13381 [6]. As we have recently characterized, this compound is a potent (nanomolar) irreversible inhibitor of AChE [7, 8]. Much like the chemical warfare agents Sarin and V/X [9], it targets the conserved Ser residue in the catalytic triad of this enzyme through phosphorylation. We recently demonstrated that bicyclic phosphonate analogs (**2a, 2b**) of cyclophostin are less potent AChE inhibitors than the natural product [8]. Monocyclic phosphonate analogs (large phosphate ring) are as potent as the corresponding bicyclic phosphonate.

Close structural relatives are the cyclipostins, long chain derivatives of cyclophostin (**3a, 3b, 4a, 4b**). These compounds isolated from bacteria were shown to be strong inhibitors of hormone sensitive lipase (HSL) [10]. This enzyme is able to catalyze the hydrolysis of mono-, di, and triacylglycerides to liberate fatty acids (Scheme 2) [11, 12]. Through insulin signaling, HSL becomes phosphorylated and translocates to lipid droplets in adipocytes [13]. Lipases are of wide interest due to their relevance to obesity and diabetes, the latter of which exhibits disturbances in both sugar and fat metabolism. There are only a few obesity drugs on the market. The most well known is Orlistat (Fig. 1), obtained from the catalytic hydrogenation of the natural product lipstatin, which targets the digestive gastric and pancreatic lipases [14]. Many more are/have been in clinical trials, and similarly a couple target lipases [3].



Scheme 1.



Scheme 2.

A number of low nanomolar HSL synthetic inhibitors have been reported (Fig. 1.; [15–24]). Very few detailed kinetic and mechanistic studies of HSL inhibitors exist [25].

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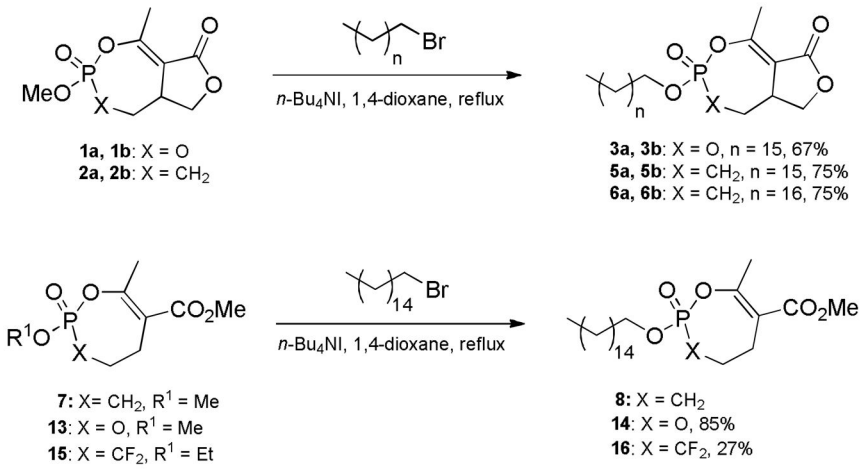
Cyclipostins represent a unique niche among HSL inhibitors because they are natural products and, by virtue of being both lipophilic and electrophilic, are substrate mimic-mechanism-based inhibitors. The long hydrocarbon tails suggest a substrate-type binding mode. Cyclophostins share the cyclic phosphate electrophilic center of cyclipostins, which have already been shown to irreversibly modify the active site Ser of AChE [8] and microbial lipases [26]. We recently reported the synthesis of cyclipostin P (**4a, 4b**) [27]. Herein we characterize the activities for two cyclipostins and their analogs against hormone sensitive lipase. To our knowledge, this represents the most detailed study of HSL inhibitors reported to date.

2. RESULTS AND DISCUSSION

2.1. Synthesis of Inhibitors

Cyclipostin R (**3a, 3b**) and the analogs used in this study were synthesized using our one-pot ester exchange process from (±)-cyclophostin **1a** and its diastereomer **1b** [27], the phosphonate analogs thereof **2a** and **2b** [7], and three monocyclic compounds **7, 13** and **15** (Scheme 3). Characterization data for the monocyclic compounds **13** and **15** appear in Supplemental Material. The syntheses these compounds will be reported in another article. Methyl esters of phosphates undergo transesterification cleanly in the presence of 5 mol% tetra-*n*-butylammonium iodide, but phosphonates and the α, α-difluorophosphonate ethyl ester **16** require 10 mol% for optimal conversion. Additional information can be found in **Experimental** and Supplementary Material.

Cyclophostin **1a 1b**, cyclipostin P **4a**, **4b** and the phosphonate analogs of cyclophostin (**5a**, **5b**, **6a**, **6b**) were prepared following previously published procedures [7, 27]. The synthesis of the monocyclic phosphonates (**7, 8, 9a, 10a, 10b, 11a, 11b, 12a, 12b**) also followed published procedures [8, 26].



Scheme 3.

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2.2. Inhibition Studies with Cyclophostin and Cyclipostin

Table 1 summarizes all of the cyclophostin and cyclipostin analogs that were tested against recombinant rat HSL. The natural product and AChE inhibitor cyclophostin **1b** is not potent against HSL (IC_{50} • 100 μM). The same is true of the synthetic isomer **1a** and phosphonate isomer **2a**. However, the cyclipostins with the same core cyclophostin structure with long carbon tails extending from the phosphate moiety (**3–6**) are more potent against HSL; a couple have IC_{50}'s near 50 nM. This indicates that the long hydrocarbon tail of the phosphate moiety is a critical part of the inhibitor for successful inactivation of rat HSL. The most potent cyclipostin is cyclipostin P *trans* (**4a**) with an IC_{50} of 25 ± 15 nM.

2.3. Monocyclic and Phosphonate Analogs

Interestingly, phosphate **14**, a monocyclic analog of cyclipostin P **(4a, 4b)** retains potency similar to the stronger diastereomer **4a** of the natural product, indicating that like cyclophostin with AChE, the integrity of the smaller lactone ring is not critical to activity. To understand the importance of the phosphate itself, both bicyclic and monocyclic phosphonates were prepared and tested. Bicyclic phosphonate analogs of cyclipostins P and R (**5a, 5b, 6a,** and **6b**) generally exhibit IC_{50}s similar to those of the weaker diastereomer phosphates (about 400 nM). The exception is **6a**, the *trans* cyclipostin P analog, which is about 10-fold less potent. The monocyclic phosphonate analog of cyclipostin P (**8**) exhibits potency similar to the weaker diastereomer of the natural product (**4b**). Interestingly, the activity difference between phosphates and phosphonates is smaller for HSL inhibitors than for AChE and cyclophostin (less than 10-fold vs. 100-fold; [28]). This further supports the contribution of the hydrocarbon chain to binding affinity.

2.4. Moving the Hydrophobic Tail

Another group of analogs extend hydrophobic tails of differing lengths (C_{10}–C_{18}) from the lactone side of the larger ring (instead of from the phosphate) (**9a**, **9b, 10a, 10b**, **11a, 11b**, **12a**, **12b**). Most of these are modest inhibitors of HSL, with IC_{50}'s ranging from 13–60 μM. Among the compounds with chain lengths of 12–18 carbon atoms, **10b**, **11b**, **12b** (*trans*) are generally 2–6 times more potent than the corresponding *cis* isomers **(10a, 11a, 12a**). Interestingly, these compounds are inhibitors of microbial lipolytic enzymes with marked stereoselectivity [26, 29].

2.5 Effect of Difluorination of Phosphonate Ring

If the inhibitor functions via electrophilicity, then introducing electron withdrawing groups into the phosph(on)ate ring should result in an increase in potency. To that end, fluorinated monocyclic monophosphate **16** was prepared. Interestingly, while its nonfluorinated relative **8** has a 0.5 μM IC_{50}, the IC_{50} for **16** is over 100 μM. Clearly other factors, perhaps binding, affect inhibitor potency.

2.6. Stereochemical Differences Among Cyclipostins and Analogs

As summarized in Scheme 1, the presence of 2 stereocenters in both cyclophostin and cyclipostins structures give rise to two sets of diastereomers: *cis* refers to the isomers in

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which the phosphate ester chain and cyclic proton are on the same side of the ring system; *trans* refers to those isomers in which these groups are on opposing sides of the ring system.

The *trans* diastereomers of cyclipostin P **4a** are the more potent, with an IC_{50} of 25 nM. The *cis* diastereomers of cyclipostin P **4b** are 16 fold weaker. Interestingly, the opposite is true of cyclipostin R: in this case, the *cis* diastereomers **3b** are similarly more potent than the *trans* **3a**. Among the phosphonate analogs of cyclipostins P and R, the *cis* diastereomers of cyclipostin P analogs (**6a, 6b**) are more potent (360 nM, 20-fold better than *trans*). Interestingly, for the phosphonate analogs (**5a, 5b**) of cyclipostin R (**3a, 3b**), the IC_{50}s for *cis* and *trans* are comparable. While it is difficult to easily rationalize why a one-carbon change in the hydrophobic tail would change diastereopreference by the enzyme, this behavior has precedent: The *cis* isomer of the C_{10} monocyclic phosphonate analog **9a** is more potent against Rv183 lipase from *Mycobacterium tuberculosis* than the corresponding C_{10} *trans* analog **9b** [26]. However, the *trans* isomer of the C_{12} analog **10b** is more potent than the corresponding *cis* isomer **10a** [26]. One possible rationalization is how hydrocarbon chains are accommodated in enzyme active sites. There is some precedent for chain length affecting the stereochemical behavior of lipases [30, 31]. Nevertheless, observing a difference in stereochemical behavior between compounds with tails of 15 and 16 carbon lengths is remarkable. Better understanding of this behavior would require a structure of HSL. Unfortunately, this enzyme has been refractory to structural biology efforts. While an HSL structural model has been constructed based on sequence homology between the crystallographically characterized brefeldin A esterase and the C-terminal domain of HSL [32], models with greater experimental support (and thus detail) would be necessary to meaningfully rationalize the IC_{50} patterns observed here.

2.7. Mechanism of Inhibition of Cyclipostin P *Trans* (4a)

As stated in the introduction, we have previously shown that cyclophostin inhibits AChE irreversibly [8]. Since the basic chemistry should be the same, we expect cyclipostin to inhibit HSL irreversibly. This clarification simplifies the kinetic analysis, since reversible and irreversible inhibitors are analyzed differently.

To confirm this, reaction timecourse data for cyclipostin P *trans* (**4a**) were fit to Eqn 1 to obtain k_{obs} and k_{-2}, the latter of which is the rate constant for the reverse reaction [8]. Sample data appear in Fig. 2A. In these fits at various inhibitor concentrations, k_{-2} averages to 0.001 min^{-1}. Thus this inhibitor can be treated as irreversible.

To dissect this behavior, residual enzyme activities were obtained as a function of inhibitor concentration and incubation time for *trans* cyclipostin P (**4a**) (Fig. 2B). The irreversible inactivation mechanism in Scheme 5 [33] was utilized. The extent of reaction, as measured by the change in cpm for the liberated radioactive free fatty acid, decreases as a function of inhibitor concentration. This was fit to Eqn. 2 to obtain k_2, the forward rate constant, and K_I, the equilibrium constant for binding, for HSL and **4a**. The values for these constants are 40 nM and 0.2 min^{-1}, respectively. Dividing the former constant by the latter yields a first order rate constant for inactivation of 5×10^6 $M^{-1}min^{-1}$. Since we are unaware of any reports of this type of data for any other HSL inhibitor, we must compare to other enzymes.

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Indeed, this rate constant is comparable to that measured for cyclophostin [27] and tabun [8] toward human AChE. However, it is 1000-fold higher (faster) than that estimated from studies of the interactions of Orlistat with lipoprotein lipase [34].

It is possible that phosphonate analogs of cyclipostin P *trans* would display different kinetic details. Comparing AChE inhibitors cyclophostin (**1a, 1b**) and their phosphonate analogs (**2a, 2b**), k_2 and k_{-2} were similar but K_I values differed by 100-fold in parallel with the corresponding IC_{50}s [27]. The HSL IC_{50}'s of the cyclipostins (**3a, 3b, 4a, 4b**) and their phosphonate analogs tested here (**3a, 3b, 4a, 4b**) are generally more similar to one another (6 to 16-fold, excluding **6a**). Thus more modest differences in kinetic parameters would be expected. It is not possible to perform analyses for additional compounds at this time.

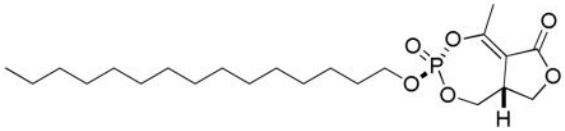
In conclusion, we have prepared and evaluated a series of cyclipostins and their analogs toward hormone sensitive lipase. The most potent of these are active in the low nanomolar range and display irreversible kinetics. SAR studies indicate that the smaller lactone ring is not essential; however the lipophilic tail is critical to activity.

3. EXPERIMENTAL

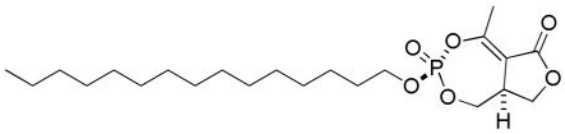
3.1. Synthesis and Characterization of Organophosphates

The preparations of (**1a**, **1b**, **2a**, **4a**, **4b**, **8**,**16**, **9a**, **10a**, **10b**, **11a**, **11b**, **12a**, **12b** have been described previously [7, 26, 27].

3.1.1. Diastereomers of Cyclipostin R (3a, 3b)—To a solution cyclophostin diastereomer **1a, 1b** (28.5 mg, 0.122 mmol) and 1-bromopentadecane (350 μL, 1.21 mmol) in dry 1,4-dioxane (0.5 mL) was added *n*-Bu_4NI (2 mg, 0.005 mmol) at room temperature. The flask was immersed in an oil bath preheated at 110 °C until the reaction was complete (TLC and ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/hexane) to give cyclipostin R **3a** (16.6 mg) and the unnatural diastereomer **3b** (9.5 mg) as white solids (67% overall).

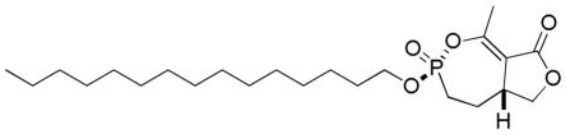


3.1.1.1. Cyclipostin R (3a, 3b). (±)-(3R(S),8aR(S))-5-methyl-3-(pentadecyloxy)-8,8a-dihydrofuro[3,4-e][1,3,2]dioxaphosphepin-6(1H)-one 3-oxide: IR (neat, ATR): 2916, 2850, 1749, 1671 $cm^{-1;}$ ^1H NMR (300 MHz, $CDCl_3$) δ 4.45 (1H, m), 4.35 (1H, td, J_{HH} = 10.8 Hz, J_{HP} = 6.2 Hz), 4.24 (2H, dt, J_{HP} = J_{HH} = 6.8 Hz), 4.15 (1H, ddd, J_{HP} = 25.6 Hz, J_{HH} = 11.2, 3.5 Hz), 3.80 (2H, m), 2.47 (3H, d, J_{HP} = 1.9 Hz), 1.72 (2H, p, J_{HH} = 6.8), 1.39 (2H, m), 1.26 (22H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 169.1, 161.8 (d, J_{CP} = 7.7 Hz), 112.1 (d, J_{CP} = 3.2 Hz), 70.4 (d, J_{CP} = 6.3 Hz), 67.6 (d J_{CP} =, 5.7 Hz), 64.4, 39.9, 32.1, 30.3 (d, J_{CP} = 6.6 Hz), 29.90, 29.88, 29.85, 29.76, 29.68, 29.58, 29.26, 25.5, 22.9, 18.4 (d, J_{CP} = 4.7 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ −8.55; HRMS (FAB, NBA, MH^+) calcd for $C_{22}H_{40}O_6P$: 431.2563, found: 431.2563.

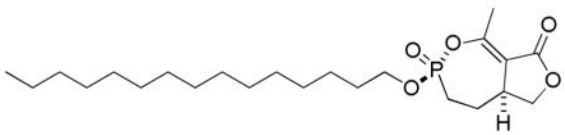


3.1.1.2. (±)-(3*R*(*S*),8aS(*R*))-5-methyl-3-(pentadecyloxy)-8,8a-dihydrofuro[3,4-e][1,3,2]dioxaphosphepin-6(1H)-one 3-oxide: IR (neat, ATR): 2914, 2847, 1745, 1666, 1289, 1242, 1204, 1070, 1034 $cm^{-1;}$ ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.46 (1H, t, J_{HH} = 9.3 Hz), 4.29 (1H, m), 4.18 (2H, m), 4.04 (2H, m), 3.79 (1H, dd, J_{HH} = 9.6, 5.7 HZ), 2.44 (3H, d, J_{HP} = 1.8 Hz), 1.73 (2H, p, J_{HH} = 6.7 Hz), 1.26 (24H, br s), 0.89 (3H, t, J_{HH} = 6.6 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 169.2, 161.6 (d, J_{CP} = 10.5 Hz), 111.0 (d, J_{CP} = 2.6 Hz), 69.9 (d, J_{CP} = 6.3 Hz), 69.5 (d, J_{CP} = 7.6 Hz), 64.7, 38.6, 32.1, 30.4 (d, J_{CP} = 6.5 Hz), 29.90, 29.88, 29.84, 29.76, 29.67, 29.58, 29.3, 25.5, 18.1 (d, J_{CP} = 5.9 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ −12.4; HRMS (FAB, NBA, MH^+) calcd for $C_{22}H_{40}O_6P$: 431.2563, found: 431.2570.

3.1.2. Phosphonate analog of Cyclipostin R and Its Diastereomer (5a, 5b)—To a solution of phosphonate analogs **2a** and **2b** (17.8 mg, 0.077 mmol) and 1-bromopentadecane (220 μL, 0.76 mmol) in dry 1,4-dioxane (390 μL) was added *n*-Bu_4NI (3 mg, 0.008 mmol). The flask was immersed in an oil bath preheated to 110 °C until the reaction was complete (TLC, ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/ hexane) giving the phosphonate analog of cyclipostin R (13.4 mg) and its diastereomer (8.9 mg) as a white solid and white semisolid, respectively (75% overall).



3.1.2.1. (±)-(3R(*S*),5a*R*(*S*))-1-methyl-3-(pentadecyloxy)-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide: IR (neat, ATR): 2915, 2849, 1742, 1662, 1246, 1036 $cm^{-1;}$ ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.50 (1H, t, J_{HH} = 9.3 Hz), 4.19 (2H, m), 3.85 (1H, dd, J_{HH} = 9.2, 6.6 Hz), 3.32 (1H, m), 2.45 (3H, s), 2.32 (1H, m), 1.89–2.13 (3H, m), 1.72 (2H, p, J_{HH} = 6.9), 1.26 (24H, br s), 0.88 (3H, t, J_{HH} = 6.6 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.1 (d, J_{CP} = 1.6 Hz), 161.0 (d, J_{CP} = 7.2 Hz), 114.5 (d, J_{CP} = 3.8), 69.9, 66.8 (d, J_{CP} = 7.3 Hz), 39.1 (d, J_{CP} = 1.0 Hz), 32.1, 30.5 (d, J_{CP} = 6.1 Hz), 29.89, 29.88, 29.86, 29.85, 29.83, 29.75, 29.7, 29.6, 29.3, 26.8 (d, J_{CP} = 136.4 Hz), 26.6 (d, J_{CP} = 6.9 Hz), 25.6, 22.9, 19.0 (d, J_{CP} = 2.3 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 23.4; HRMS (FAB, NBA, MH^+) calcd for $C_{23}H_{42}O_5P$: 429.2770, found: 429.2777



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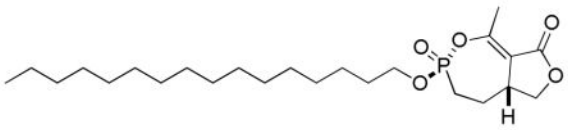
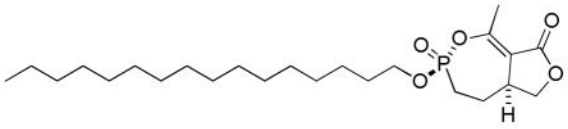
3.1.2.2. (±)-(3*R*(*S*),5aS(*R*))-1-methyl-3-(pentadecyloxy)-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide: IR (neat, ATR): 2915, 2848, 1742, 1672, 1246, 1198, 1040, 1011 $cm^{-1;}$ ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.50 (1H, t, J_{HH} = 9.3 Hz), 4.24 (1H, ddt, J_{HH} = 8.3 Hz, J_{HP} = J_{HH} = 7.7 Hz), 4.06 (1H, ddt, J_{HH} = 9.9 Hz, J_{HP} = J_{HH} = 6.9 Hz), 3.84 (1H, dd, J_{HH} = 9.2, 6.5 Hz), 3.40 (1H, m), 2.43 (3H, d, J_{HP} = 1.7 Hz), 1.80–2.40 (4H, m), 1.70 (2H, p, J_{HH} = 6.9 Hz), 1.26 (24H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.3, 160.5 (d, J_{CP} = 9.6 Hz), 113.8 (d, J_{CP} = 3.0 Hz), 69.9, 67.0 (d, J_{CP} = 7.0 Hz), 38.6 (d, J_{CP} = 2.0 Hz), 32.1, 29.89, 29.87, 29.83, 29.76, 29.68, 29.57, 29.3, 27.0 (d, J_{CP} = 7.5 Hz), 26.6 (d, J_{CP} = 134.5 Hz), 25.6, 22.9, 18.7 (d, J_{CP} = 3.5 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 19.9; HRMS (FAB, NBA, MNa^+) calcd for $C_{23}H_{41}O_5PNa$: 451.2589, found: 451.2601.

3.1.3. Phosphonate Analog of Cyclipostin P and Its Diastereomer (6a, 6b)—To a solution of phosphonate analog **2a, 2b** (10.7 mg, 0.046 mmol) and 1-bromohexadecane (140 μL, 0.46 mmol) in dry 1,4-dioxane (230 μL) was added *n*-Bu_4NI (1.7 mg, 0.0046 mmol). The flask was immersed in an oil bath preheated to 110 °C until the reaction was complete (TLC, ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/ hexane) giving the phosphonate analog of cyclipostin P (7.8 mg) and its diastereomer (5.5 mg) as a white solid and white semisolid, respectively (75% overall).



3.1.3.1. (±)-(3*R*(*S*),5aR(*S*))-3-(hexadecyloxy)-1-methyl-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide: IR (neat, ATR): 2918, 2852, 1742, 1661, 1246, 1040 $cm^{-1;}$ ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.50 (1H, t, J_{HH} = 9.3 Hz), 4.20 (2H, m), 3.86 (1H, dd, J_{HH} = 9.2, 6.6 Hz), 3.34 (1H, m), 2.46 (3H, s), 2.26–2.38 (1H, m), 1.89–2.13 (3H, m), 1.72 (2H, p, J_{HH} = 6.9 Hz), 1.26 (26H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.1, 161.0 (d, J_{CP} = 7.0 Hz), 114.5 (d, J_{CP} = 4.0 Hz), 69.9, 66.8 (d, J_{CP} = 7.1 Hz), 39.1, 32.1, 30.5, 29.89, 29.87, 29.84, 29.77, 29.70, 29.6, 29.3, 26.8 (d, J_{CP} = 136.0 Hz), 26.6 (d, J_{CP} = 7.0 Hz), 25.6, 22.9, 19.0 (d, J_{CP} = 2.6 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 23.4; HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{44}O_5P$: 443.2926, found: 443.2935.



3.1.3.2. (±)-(3*R*(*S*),5aS(*R*))-3-(hexadecyloxy)-1-methyl-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide: IR (neat, ATR): 2916, 2848, 1749, 1669, 1242, 1201, 1043, 1011; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.51 (1H, t, J_{HH} = 9.1 Hz), 4.24 (1H, ddt, J_{HH} = 9.8 Hz, J_{HH} = J_{HP} = 7.2 Hz), 4.06 (1H, ddt, J_{HH} = 10.0 Hz, J_{HH} = J_{HP} = 7.0 Hz), 3.84

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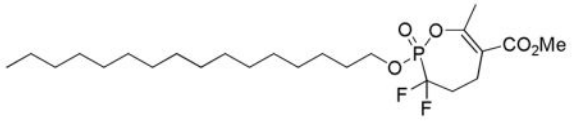
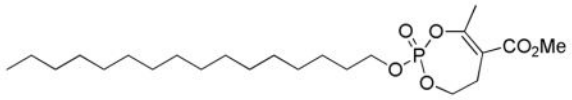
(1H, dd, J_{HH} = 9.1, 6.4 Hz), 3.41 (1H, m), 2.43 (3H, s), 1.80–2.49 (4H, m), 1.70 (2H, p, J_{HH} = 6.8 Hz), 1.32 (2H, m), 1.26 (24H, br s), 0.88 (3H, t, J_{HH} = 6.6 Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.3, 160.5 (d, J_{CP} = 9.8 Hz), 113.8 (d, J_{CP} = 3.0 Hz), 70.0, 67.1 (d, J_{CP} = 7.1 Hz), 38.6 (d, J_{CP} = 2.2 Hz), 32.1, 30.6 (d, J_{CP} = 5.9 Hz), 29.92, 29.90, 29.88, 29.87, 29.84, 29.76, 29.68, 29.58, 29.3, 27.0 (d, J_{CP} = 7.9 Hz), 26.6 (d, J_{CP} = 134.7 Hz), 25.6, 22.9, 18.7 (d, J_{CP} = 3.2 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 20.0; HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{44}O_5P$: 443.2926, found: 443.2919



3.1.4. Methyl 3,3-difluoro-2-(hexadecyloxy)-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide (16)—To a solution of cyclic phosphonate **15** (26 mg, 0.091 mmol) in dry 1,4-dioxane (0.45 mL) was added 1-bromohexadecane (280 μL, 0.92 mmol) and *n*-Bu_4NI (3.4 mg, 0.0092 mmol). The flask was immersed in an oil bath preheated to 110 °C until the reaction was complete (TLC, ^{31}P NMR analysis). The mixture was cooled to room temperature and concentrated *in vacuo*, and the residue was chromatographed on C_{18} reverse phase silica gel (CH_3OH/H_2O) to give **16** (12 mg, 27%) as a white solid. IR (Neat, ATR) 2963, 2917, 2850, 1717, 1661, 1292, 1271, 1095, 1011 $cm^{-1;}$ ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.34 (2H, dt, J_{HH} = J_{HP} = 7.0 Hz), 3.79 (3H, s), 2.65–2.79 (1H, m), 2.45–2.59 (1H, m), 2.40 (3H, s), 2.17–2.39 (2H, m), 1.78 (2H, p, J_{HH} = 6.9 Hz), 1.26 (26H, br s), 0.89 (3H, t, $J_{H,H}$ = 6.8 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 167.4, 159.2 (d, J_{CP} = 8.1 Hz), 120.4 (td, J_{CF} = 261.7 Hz, J_{CP} = 205.1 Hz), 120.1 (d, J_{CP} = 5.6 Hz), 69.9 (d, J_{CP} = 7.0 Hz), 52.3, 35.3 (td, J_{CF} = 20.5 Hz, J_{CP} = 11.4 Hz), 32.152, 30.6 (d, J_{CP} = 5.6 Hz), 29.90, 29.88, 29.84, 29.74, 29.66, 29.57, 29.2, 25.4, 22.9, 21.4, 20.2 (dd, J_{CF} = 9.1 Hz, 3.5 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 1.4 (dd, J_{PF} = 111.2 Hz, 100.6 Hz); ^{19}F NMR (282.2 MHz, $CDCl_3$): δ −109.2 (dd, J_{FF} = 290.1 Hz, J_{FP} = 111.2 Hz), −106.1 (dd, J_{FF} = 290.1 Hz, J_{FP} = 100.3 Hz); HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{44}F_2O_5P$: 481.2894, found 481.2900.



3.1.5. Methyl 2-(hexadecyloxy)-4-methyl-6,7-dihydro-1,3,2-dioxaphosphepine-5-carboxylate 2-oxide (14)—To a solution of phosphate **13** (11 mg, 0.047 mmol) and 1-bromohexadecane (175 μL, 0.473 mmol) in dry 1,4-dioxane (235 μL) was added *n*-Bu_4NI (1 mg, 0.003 mmol). The flask was immersed in an oil bath preheated to 110 °C until the reaction was complete (TLC, ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/ hexane) giving **14** (18 mg, 85%) as a colorless oil. IR (neat, NaCl): 2924, 2854, 1723, 1645, 1301, 1104, 1011 $cm^{-1;}$ ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.37 (1H, m), 4.17 (3H, m), 3.77 (3H, s), 3.00 (1H, ddd, J_{HH} = 15.5, 8.8, 3.2 Hz), 2.84 (1H, ddd, J_{HH} = 15.6, 5.9, 3.6 Hz), 1.71 (2H, p, J_{HH} = 6.7 Hz), 1.26 (26H, br s), 0.88 (3H, t, J_{HH} = 6.7

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Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 167.3 (d, J_{CP} = 2.0 Hz), 161.1 (d, J_{CP} = 9.6 Hz), 115.6 (d, J_{CP} = 4.1 Hz), 69.3 (d, J_{CP} = 6.6 Hz), 68.3 (d, J_{CP} = 6.6 Hz), 52.2, 32.1, 30.5 (d, J_{CP} = 6.6 Hz), 29.90, 29.87, 29.84, 29.76, 29.69, 29.55, 29.3, 28.5, 25.6, 22.9, 20.5 (d, J_{CP} = 4.0 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ −10.8; HRMS (FAB, NBA, MH^+) calcd for $C_{23}H_{44}O_6P$: 447.2876, found 447.2864.

3.2. Enzyme Studies

3.2.1. Protein Expression—The *Spodoptera frugiperda* (Sf9) cells were grown in TNM-FH medium supplemented with 10% heat inactivated FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 27°C. The cells were maintained in T-150 flasks (Fisher Scientific) and subcultured in 1:3 ratio whenever they reached 90% confluency.

The rat hormone-sensitive lipase gene was a kind gift from Dr. Fredric Kraemer. The gene was cloned into a pcDNA3 vector using *HindIII* and *XbaI* sites. The HSL gene was subcloned in the pAcHLT-A baculovirus transfer vector (BD Biosciences), having N-terminal 6X-His Tag gene at a SmaI blunt cloning site. The pAcHLT-A-HSL construct was transformed into TOP10F′ *E. coli* competent cells for large scale DNA production.

Recombinant baculovirus containing the HSL gene was prepared using the HSL gene from the pAcHLT-HSL plasmid and the linearized Baculogold DNA (BD Biosciences). The transfection into Sf9 insect cells was accomplished by utilizing the Ca^{II}-phosphate precipitation method using a BaculoGold Transfection Kit (BD Biosciences). The successful formation of recombinant baculovirus containing the HSL gene was confirmed by Western Blot analysis using an anti-rabbit HSL polyclonal antibody as the primary antibody (Cayman Chemicals Ltd.), and anti-rabbit horseradish peroxidase (Promega) as the secondary antibody on the virus-infected cell lysate [35, 36].

For high titer rat HSL baculovirus production, transfected SF9 cells were left to grow for 144 hours (6 days). The media from transfected SF9 cells was collected and sent to Allele Biotechnology for virus amplification (1 e8 pfu/ml). For protein production, 4 e7 SF9 cells in T-150 flasks (Fisher) (70 % confluent) were infected with recombinant baculovirus at 8 m.o.i. SF9 cells were collected after 72 hour post infection and centrifuged at 1,500 g for 20 minutes and stored at −80°C.

3.2.2. Protein Purification—In a protocol adapted from the literature [32], cell pellet was homogenized in 75 mM Tris, 10% glycerol, 1 mM DTT and 1% $C_{13}E_{12}$ (poly (ethylene glycol)(12) tridecyl) (Sigma) at pH 8.0 at 4°C and supplemented with 0.2 μM bestatin hydrochloride, 0.01 μM E-64 and 10 μM phosphoramidon disodium salt to inhibit proteolytic degradation. The solution volume was adjusted to 15 ml of buffer per gram of pellet. The resulting suspension was sonicated on ice using a Branson Sonifier 250 sonicator for 2 min at setting 2, then 2 min at setting 4 with 30 sec intervals between each minute of sonication. This solution was centrifuged 11,000 g at 4°C for 30 min. Supernatant containing 6X-His tag rat HSL incubated Ni-NTA beads for 1.5 hour at 4 °C. This suspension was washed twice with 1 mL of elution buffer containing 75 mM Tris, 0.3 M NaCl, 1 mM DTT, 10 % glycerol, 0.01 % C_8E_4 (tetrathylene glycol monooctyl ether) (Sigma) pH 8.0 to wash away nonspecifically bound proteins. Pure HSL was eluted with an

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imidazole gradient (5–400 mM) in elution buffer. Fractions were collected and evaluated by SDS PAGE (10%). The most concentrated and most pure fractions were combined and dialyzed using a Slide-A-lyzer (Pierce) against 1 L of elution buffer without imidazole. The concentration of recombinant rat HSL was measured using a Shimadzu UV-1800 spectrophotometer with an extinction coefficient of 52090 M^{-1} cm^{-1} at 280 nm [37].

3.2.3. HSL Lipase Assay—Oleic acid release from triolein embedded in liposomes was used to monitor activity of HSL. In protocol adapted from the literature [38, 39], liposomes consisted of 200 μM phosphatidylcholine and phosphatidylinositol (3:1 w/w) and 500 μM cold triolein and 120 μL of ^{3}H triolein per 20 mL. All of these components were dried from chloroform under vacuum before resuspension in assay buffer (25 mM Tris, 150 mM NaCl, 0.2 mM EDTA, and 1 mM β-mercaptoethanol, pH7.4). Sonication was performed on ice for 2 min at setting 2 and 2 minutes at setting 4 with 30 second pause between each minute. 0.2 % BSA was added to the reaction.

Reactions were performed at 37°C with HSL (20–100 nM as needed) and followed for 30 min by removing 120 μL of the reaction mixture at various time points (either every 5 or 10 min for 6–8 total points) and adding 50 μL stop buffer (50 mM each of K_2CO_3, K_3BO_3, KOH, pH 10). Liberated, radioactively labeled oleic acid was extracted twice with 100 μL of methanol: chloroform: heptanes (1.41: 1.25:1, v/v) [38]. The aqueous layer was then assessed by scintillation counting. These data were used to calculate a raw rate (cpm/min).

3.2.4. Inhibition Studies—Inhibitor solutions were prepared similar to those above for triolein. A separate liposome solution was prepared for each inhibitor concentration used. For IC_{50} determinations, enzyme was preincubated for 30 min on ice with inhibitor-embedded liposomes of the same component concentrations (PC, PI and inhibitor) as in the assay. Triolein was omitted in the preincubation mixture. At 10 min intervals (6–8 timepoints), the reaction mixture was assayed. IC_{50} was determined as amount of inhibitor required to reduce rat HSL activity to 50 %. Each IC_{50} was performed in triplicate.

3.2.5. Determination of Rate Constants for Binding and Inactivation—We began with the general reaction scheme:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E.I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI$$

Scheme 4.

where K_I represents the dissociation equilibrium constant for the inhibitor, k_2 is the forward rate constant for inactivation of the enzyme and k_{-2} is the reverse rate constant for inactivation. In many irreversible inhibition reactions, k_{-2} is neglected [33]. To determine if

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that was appropriate here, kinetic data at a few representative inhibitor concentrations were fit to the equation:

$$A_t = A_0[(1 - k_{-2}/k_{obs}) * e^{-(k_{obs} * t)} + k_{-2}/k_{obs}] \quad (1)$$

where A_t equals the fraction of enzymatic activity remaining following pre-incubation time (t), A_0 represents the enzymatic activity at time zero (i.e., the initial activity), and k_{obs} is the observed rate of decay. k_{-2} fit to essential zero (Fig. 2), which simplifies the model to [40, 41]:

$$E + I \underset{}{\overset{K_I}{=}} E.I \xrightarrow{k_2} EI$$

Scheme 5.

In this analysis, HSL was not preincubated with inhibitor on ice, but was added immediately to substrate-inhibitor mixture at 37° C and timepoints taken every 3–5 minutes for 30 minutes. As inhibitor concentration is increased, the extent of reaction (as measured from scintillation counting of extracted samples) decreases. This is converted to a relative normalized activity and can be expressed as a function of k_2 and K_I:

$$\text{Relative activity} = k_2 * [I]/(K_I + [I]) \quad (2)$$

Data were fit to this equation using Kaliedagraph to yield K_I and k_2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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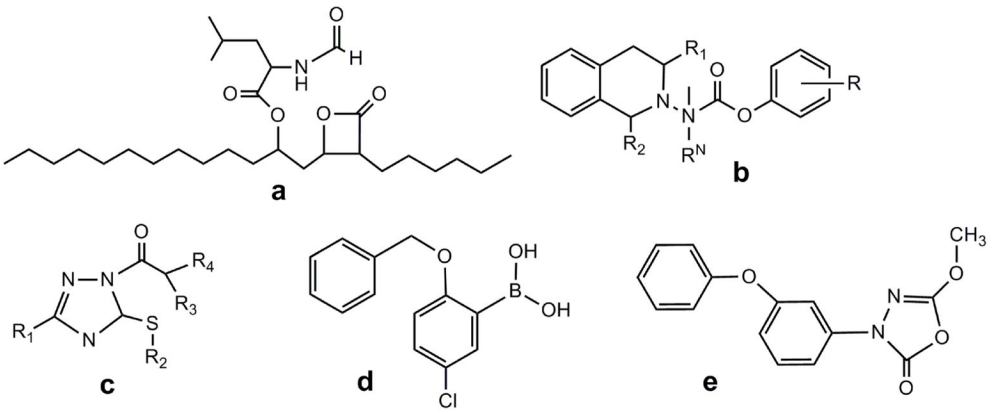

Fig. 1. Other Representative Lipase Inhibitors

(a) pancreatic lipase inhibitor Orlistat [42]; HSL inhibitors (b) carbazates [18]; (c) carbamoyl triazole [17]; (d) boronic acids [19]; (e) 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-one [24, 25].

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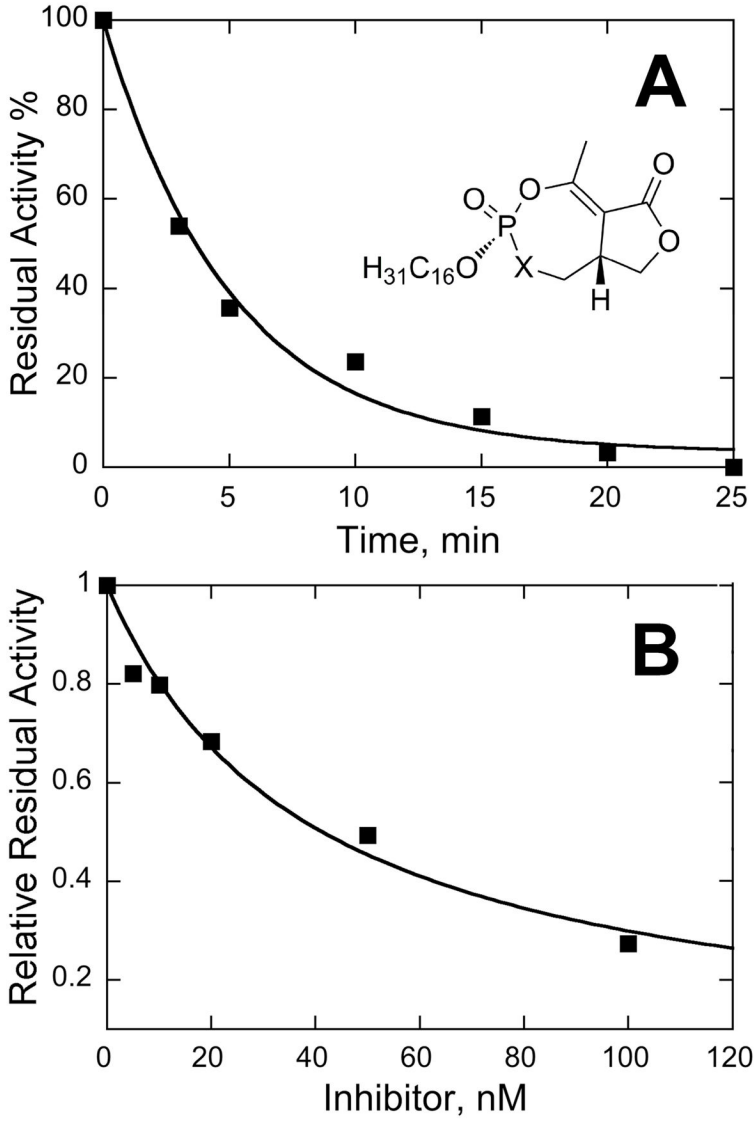

Fig. 2. Kinetic Analysis with of Cyclipostin P *Trans* (4a)

A: Timecourse for HSL activity in the presence of 10 nM **4a**, 80 nM HSL. Data were fit to Eqn 1 to demonstrate that k_{-2} is essentially zero. See text for details. **B:** Relative residual HSL activity as a function of cyclipostin P *trans* (**4a)** concentration. Data were fit to Eqn 2 to yield K_I of 40 nM and k_2 of 0.2 min^{-1}.

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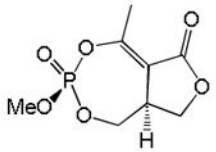
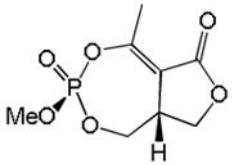
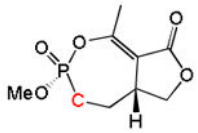
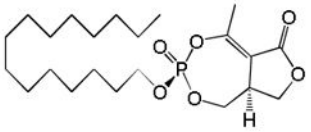
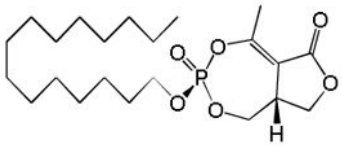
Table 1

Summary of HSL IC_{50}s of Cyclipostins and Analogs[a]

Compound	Structure	Tail	Diastereomer	IC_{50} [μM]	Published synthesis
Synthetic Cyclophostin **1a**		1	*Trans*	>100	[27]
Natural Cyclophostin **1b**		1	*Cis*	>100	[27]
Cyclophostin Phosphonate analog **2a**		1	*Trans*	100	[7]
Cyclipostin R **3a**		15	*Trans*	0.9±0.9	This work
Cyclipostin R **3b**		15	*Cis*	0.075±0.025	This work

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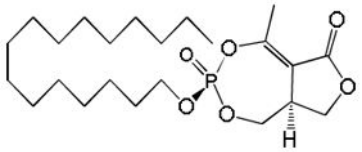
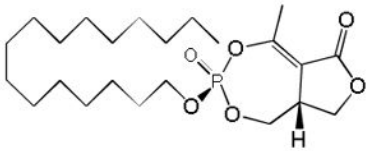
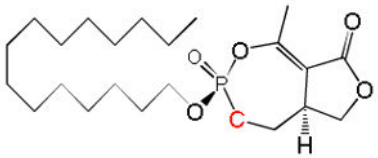
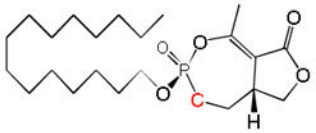
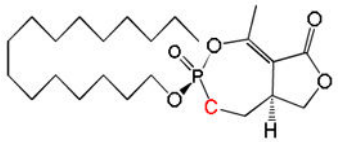
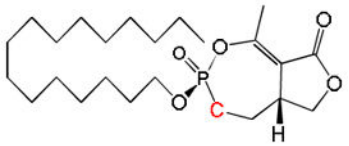
Compound	Structure	Tail	Diastereomer	IC_{50} [μM]	Published synthesis
Cyclipostin P **4a**		16	*Trans*	0.025±0.015	[27]
Cyclipostin P **4b**		16	*Cis*	0.42±0.16	[27]
Cyclipostin R Phosphonate **5a**		15	*Trans*	0.45 ± 0.25	This work
Cyclipostin R Phosphonate **5b**		15	*Cis*	0.47 ± 0.38	This work
Cyclipostin P Phosphonate **6a**		16	*Trans*	6.9 ± 2.4	This work
Cyclipostin P Phosphonate **6b**		16	*Cis*	0.36 ± 0.34	This work

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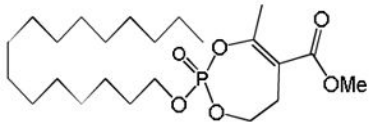
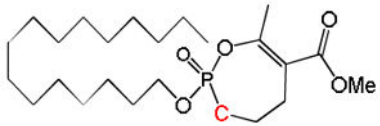
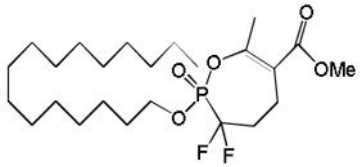
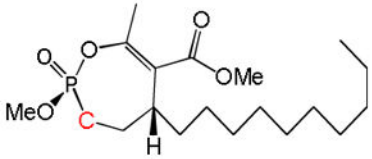
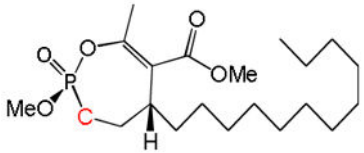
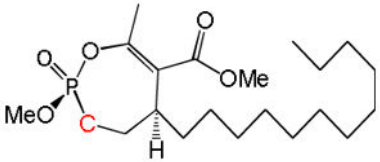
Compound	Structure	Tail	Diastereomer	IC_{50} [μM]	Published synthesis
14		16		0.06±0.02	This work
8		16		0.54±0.4	[26]
16		16		>100	This work
9a[a]		10	*Cis*	40	[26]
10a		12	*Cis*	35	[26]
10b		12	*Trans*	13.5	[26]

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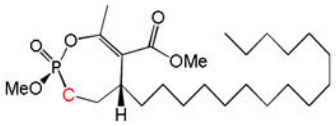
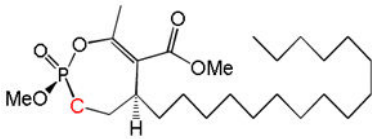
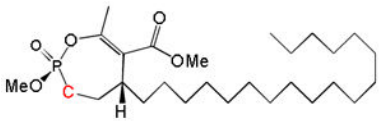
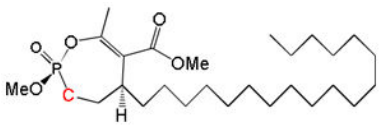
Compound	Structure	Tail	Diastereomer	IC_{50} [μM]	Published synthesis
11a		16	*Cis*	~60	[26]
11b		16	*Trans*	~10	[26]
12a		18	*Cis*	>100	[26]
12b		18	*Trans*	~27	[26]

[a] IC_{50}s, the concentration at which the enzyme is inhibited 50% of the control activity, was obtained using triolein as a substrate as described in Experimental. Graphical abstract