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Thioxophosphoranyl aryl- and heteroaryloxiranes as the representants of a new class of metallocarboxypeptidase inhibitors

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Abstract—A novel and potent family of metallocarboxypeptidase inhibitors based on thioxophosphoranyl oxiranes is presented. These compounds bear aryl or heteroaryl substituents with *trans*-stereochemistry with respect to the phosphorylated group and they have been synthesized by the addition of [bis(diisopropylamino)phosphino](trimethylsilyl)carbene to the corresponding aldehydes and the subsequent thiolation of the phosphine. These oxiranes contain a tetrahedral P atom harboring shielded *N*,*N*-groups. The screening of their biological activity as metallocarboxypeptidase inhibitors and some structural studies, as well as full experimental details for the new compounds, is disclosed. Thus, from the analysis of their activity against the prototypical metallocarboxypeptidases A and B (CPA and CPB), we have observed that hydrophobic phosphorylated oxiranes perform better as CPB inhibitors, reaching K_i values comparable to classical synthetic carboxypeptidase inhibitors. X-ray diffraction analysis revealed that the packing in the structure of one phosphorylated oxirane is mediated mainly by hydrophobic contacts and that the *N*,*N*-groups are highly flexible. Consequently, phosphorylated oxiranes might constitute an attractive material for subsequent improvements in the design of novel inhibitors against human proteolytic enzymes with enhanced oral availability.

1. Introduction

The search for drugs targeted to proteases is an extremely fertile field as suggested by the current developments,[1] and much more progress would be attained if the bioavailability problem associated to most new designs could be conveniently tackled.[2]

Phosphorylated compounds are widely used in the design of enzyme inhibitors. This class of products bear charged groups that endow them with high affinity toward the biological target. In particular, phosphorus (V) containing organic compounds have attracted much attention as inhibitors of metalloproteases because this functionality can yield a variety of metal-coordinating possibilities. The phosphorus-containing moiety provides strong binding to the metal ion at the active site in metalloenzymes, while the rest of the molecule accounts for selectivity issues. In the case of the model metalloprotease carboxypeptidase A (CPA), the phosphonic and thiophosphonic class of inhibitors have been considered as multisubstrate analogues, or as transition-state mimetics, due to the presence of a tetrahedrally hybridized phosphorus atom at the position occupied by either a trigonal planar carbon atom in the ground state of the substrate or a tetrahedrally hybridized carbon atom in the transition state. Analysis of the mode of binding of these inhibitors has provided a more profound knowledge on the catalytic mechanism of CPA in particular and of mechanistically related enzymes in general.[3]

Probably, the first examples of phosphorylated organic compounds acting as CP inhibitors are the phosphoramidates **I**–**III** (Fig. 1). Their design can be exemplified by the *N*-phosphorylated amino acid phosphoryl-L-phenylalanine, P-Phe-OH, **I** (R = OH), which fullfills the requirements of strong chelation of the active site zinc atom by the phosphoryl group and a hydrophobic interaction between the side chain of the P1′ amino acid res-

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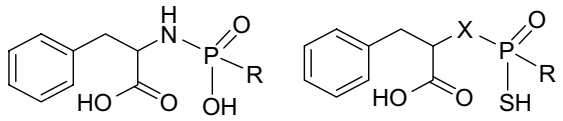


I, R = OH, CH_2NHCbz **II**, X = CH_2, R = OEt

III, X = O, R = OCH_2Ph

Figure 1. Structure of some representative CP inhibitors.

idue and the S1′ subsite (the specificity pocket) of the enzyme. Thiophosphorylated compounds such as **II**[4] and **III**[5] have also been shown to inhibit CPA. All these compounds resemble the structure of benzylsuccinic acid, a standard inhibitor to CPA and, not surprisingly, display similar inhibitory potency. Remarkably, an earlier study showed that *O*,*O*-disubstituted *N*-phosphoryl derivatives of amino acids did not inhibit CPA even at high concentrations.[6] This strongly suggests that a phosphoryl group accommodating free oxygen atoms is required for binding and that blocking the phosphoryl oxygens eliminated the inhibition.

From the mechanistic standpoint, much of our knowledge on the catalytic mechanism of metalloenzymes is based on that of CPA. This knowledge was exploited in the design of potent CPA inhibitors in the form of transition-state analogues containing a phosphorus center.[7]

Thus, *N*-[[[(benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine (ZG^PP), **I** (R = CH_2NHCbz), the phosphonamidate analogue of the CPA substrate carbobenzoxyglycyl-L-phenylalanine, represents the first of a series of extremely potent CPA inhibitors.[3] Further achievements within this series led to phosphonate tripeptides, in which the last amide bond is replaced by a phosphorus-containing moiety. These compounds can reach K_i values in the femtomolar range[8–10] and their crystal structures in complex with CPA support the notion of their behavior as transition-state analogues.[11,12] The design of tripeptide phosphonamidates has inspired a series of tight-binding inhibitors to human plasma CPB (alias CPU or TAFI),[13] a protein that belongs to the CPA family and is regarded as a target for therapeutic intervention because of its involvement in blood hemostasis.

A highly desirable goal of our approach is to search for a suitable solution to the design of compounds that could overcome the problem of poor oral availability associated to phosphorylated drugs. Since phosphorus-containing compounds have been shown to interact primarily with the zinc at the enzymes' active site, we wished to investigate the effects of a complete shielding of the charges in the phosphonate functionality on the binding affinity. Therefore, a discovery program was launched in search of novel phosphorus-containing scaffolds, such as the phosphorylated oxiranes. These compounds have displayed relevant activities as antibacterial agents[14–16] and as antibiotics. Among the latter, phosphomycin, (1*R*,2*S*)-epoxypropylphosphonic acid (Fig. 2), is the most prominent instance.[17,18]

Starting from an easy and stereoselective synthetic strategy to prepare differently substituted phosphoranyl oxiranes bearing alkyl[19,20] and aryl[21] substituents, we decided to extend this methodology to the synthesis of new heteroaryl substituted epoxides and to test the inhibitory activity toward CPA and CPB of some representants of these families. From the initial screening, we found a series of phosphorus-containing oxiranes which displayed biological activity against CPA and CPB, the prototypical metallocarboxypeptidases belonging to the MC clan of proteases.[22] Thus, we report herein on the synthesis, the identification, and the biological characterization of a new class of metallocarboxypeptidase inhibitors, the thioxophosphoranyl aryl- and heteroaryloxiranes.

2. Results and discussion

2.1. Chemistry: synthesis of the thioxophosphoranyl oxiranes

Oxiranes that contain aromatic rings bearing electron-withdrawing (NO_2, CF_3) or electron-releasing (OMe, Me) substituents as well as several heterocycle moieties were envisioned as representants of the thioxophosphoranyl aryl- and heteroaryloxiranes. Compounds **3a–d** were prepared according to the protocol previously developed in our laboratory[19–21] that involves the stereoselective addition of nucleophilic phosphinocarbene **2**,[23] resulting from photochemical decomposition of the precusor diazoalkane **1**, to aldehydes followed by the thiolation of the produced phosphinoepoxide with elemental sulfur (Scheme 1). This methodology has been successfully applied to the synthesis of the new heteroaryloxiranes **3e–k**, which were isolated in 56–92% yield. The reaction progress was monitored by ^{31}P NMR spectroscopy following the disappearance of the carbene signal of **2** ($\delta = -46$). Thiolation was achieved in about 30 min, and the corresponding epoxy thioxophosphonamides **3e–k** were characterized by chemical shifts at 83–86 ppm. No significant differences were observed in the behavior of heteroaromatic aldehydes. *trans*-Stereochemistry was assigned by comparison with other previously synthesized epoxides whose relative configuration was assigned by X-ray diffraction structural analysis.[19–21] The $^3J_{P-H}$ coupling constants for compounds **3e–k** were between 7.5 and 9.0 Hz, in good agreement with known derivatives.

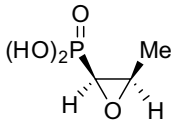
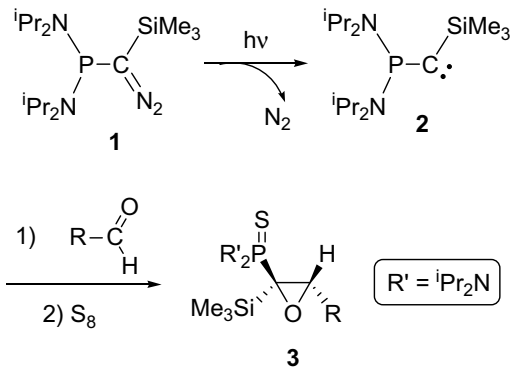


Figure 2. The antibiotic phosphomycin.



3a: R = 4-NO_2-phenyl
3b: R = 4-CF_3-phenyl
3c: R = 4-MeO-phenyl
3d: R = 4-Me-phenyl
3e: R = 2-pyridyl
3f: R = 3-pyridyl
3g: R = 4-pyridyl
3h: R = 2-thienyl
3i: R = 3-thienyl
3j: R = 2-furyl
3k: R = 3-furyl

Scheme 1.

2.2. Biology: inhibition of carboxypeptidases

The enzyme inhibition assays were performed applying the equation of Cheng and Prussoff,[24] using a fixed concentration of substrate and variable concentrations of inhibitor (usually five concentrations in the range 100 nM–500 μM). A nonlinear global fit to a competitive one-site model gave the best fitting of the experimental observations. Figure 3 shows the results for the two most potent inhibitors against CPB. The model and the experimental data were in excellent agreement ($R^2 > 0.95$) for all the assayed compounds.

The K_i values obtained for the 11 thioxophosphoranyl aryl- or heteroaryloxiranes acting against two prototypical metallocarboxypeptidases (bovine CPA and human CPB) are shown in Table 1. The capacity of thioxophosphoranyl aryl- and heteroaryloxiranes to act as inhibitors of both CPA and CPB is unprecedented, since none of the previously known *O*,*O*-disubstituted phosphoramidates were able to inhibit CPA.[6] A further unsuspected feature is that the thioxophosphoranyl aryl- and heteroaryloxiranes perform better as CPB inhibitors. CPB and CPB-like enzymes like CPU/TAFI have a preference to excise C-terminal basic lysine or arginine residues, whereas CPA cleaves substrates ending with aromatic and aliphatic residues.[25] In fact, all the thioxophosphoranyl oxiranes tested in this work are potent CPB inhibitors, the best examples belonging to the aryl series (**3a**–**d**), with slight differences probably caused by the different substituents of the phenyl ring. Thus, there seems to be a preference for groups containing an oxygen atom, as evidenced by **3a** and **3c**. The pyridine series (**3e**–**g**) ranks next, with best inhibitory potency with the nitrogen in the 4-position. The heteroaryl oxiranes-containing a thienyl moiety (**3h**–**i**) seem to be more potent than those containing a furyl ring (**3j**–**k**).

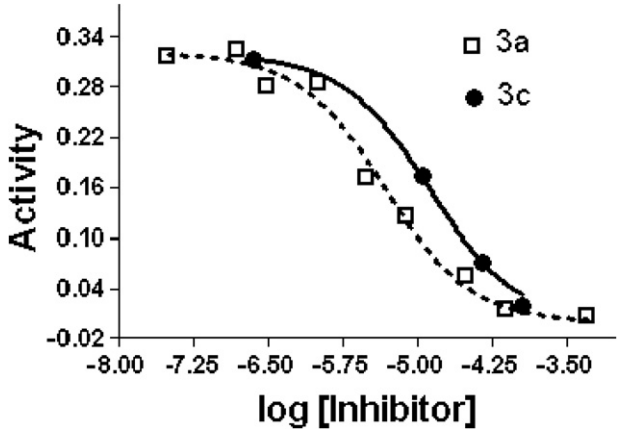


Figure 3. Plot of CPB activity, measured as the slope of the absorbance change vs time, as a function of inhibitor concentration for compounds **3a** and **3c**.

Compounds that selectively interact with one type of protease are interesting from the point of view of drug design, and specially in the case of CPB-type metallocarboxypeptidases as most enzymes of this subfamily are involved in physiologically relevant processes like the maturation of hormones or neuropeptides or, as commented above, in maintaining blood homeostasis.[25] The inhibitory potency of oxiranes **3a**–**k** against CPB is comparable to the phosphoramidates containing a free phosphoryl group. However, the structure of the former is clearly different from the known CPB peptide substrates, and this is probably one of the reasons why there are several orders of magnitude poorer CP inhibitors as compared to the tripeptide phosphonates. This fact might suggest that, although the phosphorus-containing moiety is disubstituted, and its capability to coordinate to the zinc ion is thus diminished in comparison with a free group, the substitutents have a non-deleterious effect on binding.

It should be added that, although no systematic screening of our series of compounds against other families of proteases was undertaken, a control performed using the model serine protease trypsin as a target showed no inhibitory capacity for any of the compounds at any of the assayed concentrations.

2.3. X-ray crystallography: structure of oxirane 3d

Although attempts to crystallize CPA and CPB in complex with our small-molecule inhibitors were not successful, a large single crystal grew in a drop containing CPB and inhibitor **3d**. After physically treating the crystal to rule out that it was not of protein content, it was subjected to X-ray diffraction. The subsequent structural analysis revealed that it corresponded to none of the possible inorganic components in the crystallization solution (acetate, cacodylate, PEG, or Tris) but instead to compound **3d**, the packing structure of which is shown in Figure 4.

Table 1. Biological activity of thioxophosphoranyl aryl- or heteroaryloxiranes **3a–k**

Compound	K_i (μM)	
	CPA	CPB
3a	46.0 (9)	1.06 (0.15)
3b	300.0 (20)	3.9 (0.6)
3c	70.0 (14)	3.0 (0.4)
3d	43.5 (8)	9.1 (2.3)
3e	53.3 (8.5)	15.1 (2.2)
3f	26.2 (4.5)	16.7 (2.4)
3g	29.6 (4.5)	9.7 (1.5)
3h	150.0 (18)	7.2 (2.1)
3i	62.0 (14)	9.2 (2.6)
3j	74.0 (16)	23.5 (3.9)
3k	124.0 (22)	16.7 (3.9)

Values inside parentheses indicate the standard error of the mean (SEM).

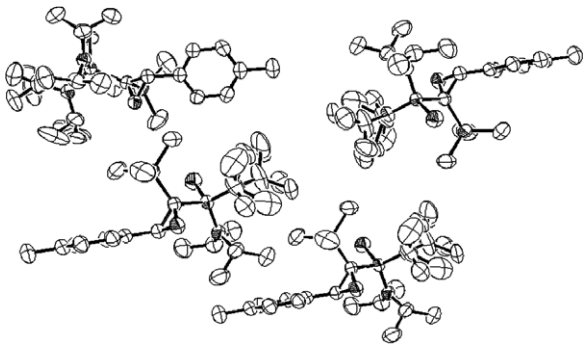


Figure 4. Crystal packing for oxirane **3d** as determined by X-ray structural analysis.

The crystal structure of **3d** is different from that of the published phenyl compound.[21] One of the diisopropylamino groups is disordered and was refined using a two sites model with a 0.60:0.40 occupancy ratio. The disorder is reflected in the large atomic displacement parameters of all the carbon atoms in this group. The possible H-bonds that **3d** can form are all intramolecular, and no electrostatic intermolecular interactions can be detected. Furthermore, the packing diagram revealed that **3d** associates in the unit cell mainly by the interactions mediated by the hydrophobic groups. The disordered diisopropyl group is located in a hydrophobic environment close to the phenyl ring. The high flexibility of the diisopropyl moiety might allow for the adoption of different conformations in accordance with the environment with which it is in contact. This can have important consequences, for example, upon binding the active site of an enzyme one might speculate that steric strains introduced by the bulky phenyl substitutents in the *O*,*O*-disubstituted phosphoramidates would impair the binding of the compound. By contrast, our *N*,*N*-disubstituted compounds harbor flexible groups that can be better adapted to fit in a hydrophobic pocket at the active site of the enzyme.

3. Conclusion

We have found a novel and potent family of metallocarboxypeptidase inhibitors based on oxiranes bearing a bis(diisopropylamino) thioxophosphoranyl group and aryl- or heteroaryl substituents with *trans*-stereochemistry. Our results show, for the first time, that a fully shielded P moiety might act as an inhibitor of a metal-dependent enzyme. Regarding CPs, it was also unanticipated that the hydrophobic phosphorylated oxiranes perform better as inhibitors toward CPB, a protease with preference to cleave residues with basic character. Phosphorylated oxiranes can be regarded as a starting point in the design of novel inhibitors against human CPB or a more relevant pharmaceutical target like the CPB-like variant plasma CPU/TAFI. The benefits of our approach include the versatility of the scaffold, its low molecular weight, and the possibility to control the hydrophilic nature without compromising their activity. This can be devised by choosing the appropriate pattern of substitutions, for example, by screening different groups at the oxirane ring or different *N*-substituents in the amino phosphine functions. Structural factors controlling the efficiency in the binding to the enzyme are under active investigation in our laboratories.

4. Experimental

4.1. General procedures and materials for the biological experiments

Bovine pancreatic CPA was from Sigma. Human CPB was prepared as published.[26,27] Enzyme concentrations for the kinetic studies were kept fixed at typically 5–50 nM. Magnesium acetate (Merck), sodium cacodylate, Tris (Tris = 2-amino-2-(hydroxymethyl)propane-1,3-diol), and PEG 8000 (Polyethyleneglycol) were from Sigma. The substrates, *N*-(4-methoxyphenylazoformyl)-Phe-OH (Aaf-Phe-OH) and *N*-(4-methoxyphenylazoformyl)-Arg-OH (Aaf-Arg-OH) were from Bachem (Switzerland). The K_m values for Aaf-Phe-OH and Aaf-Arg-OH substrates for CPA and CPB are 100 and 60 μM, respectively. The final Aaf-Phe-OH and Aaf-Arg-OH concentrations used were 100 and 200 μM, respectively. The experimental assays were performed at 50 mM Tris, 0.5 M NaCl, pH 7.5 buffer and 20 mM Tris, 0.1 M NaCl, pH 7.5 buffer for CPA and CPB, respectively. The initial velocity measurements were performed at least per triplicate at room temperature in a Victor3 (PerkinElmer, USA) microtiter plate reader. The changes in absorbance were followed continuously at 340 nm. The GraphPad Version 5.0 package (www.graphpad.com) was used to process the data.

4.2. Synthesis of thiophosphoranyloxiranes (3a–k)

4.2.1. General procedures and materials. All the manipulations were performed under an inert atmosphere of nitrogen by using standard Schlenk techniques. Dry, oxygen-free solvents were employed. Carbene **2** was prepared according to Ref. 1. All employed aldehydes were distilled before each reaction. ^{31}P NMR downfield chemical shifts are expressed with a positive sign, in ppm, relative to external 85% H_3PO_4. Microanalysis of the synthesized compounds used to afford erratic results

since the combustion of carbon was systematically incomplete. Purity criterion was assessed from cut-range melting points and homogeneity of ^{31}P, ^{1}H, and ^{13}C NMR spectra of these products.

As a general procedure for the synthesis of the thiophosphoranyloxiranes, 0.7 mmol of the dried and freshly distilled aldehyde was added to a solution of carbene **2**[23] (0.7 mmol) in pentane (3 mL) and the resultant solution was stirred at room temperature for 10 min under nitrogen atmosphere. Then an excess of elemental sulfur was added and the mixture was stirred for 30 min. The solvent was removed at reduced pressure and the residue was chromatographed on neutral silica gel (9:1 hexane/ether) to afford the pure product.

The new heteroaryl oxiranes **3e**–**k** were fully characterized as follows.

4.2.2. (2*RS*,3*RS*)-3-(2-Pyridyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3e). 77% Yield. Crystals, mp 137–139 °C (from methanol). ^{31}P NMR (101.2 MHz, acetone-d_6): δ 82.1. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.06 (s, 9H, Si(CH_3)$_3$), 1.40–1.51 (m, 24H, (CH_3)$_2$CHN–), 3.97–4.19 (m, 4H, (CH$_3$)$_2$CHN–), 4.53 (d, $^{3}J_{P-H}$ = 9.0 Hz, 1H, H$_3$), 7.37 (m, 2H, H$_{3'}$ and H$_{5'}$), 8.60 (m, 2H, H$_{4'}$ and H$_{6'}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 1.6 (Si(*C*H$_3$)$_3$), 24.1–25.1 ((*C*H$_3$)$_2$CHN–), 48.3 (d, $^{2}J_{C-P}$ = 5.7 Hz, (CH$_3$)$_2$*C*HN), 49.5 (d, $^{2}J_{C-P}$ = 5.2 Hz, (CH$_3$)$_2$*C*HN), 61.7 (d, $^{1}J_{C-P}$ = 70.1 Hz, C$_2$), 63.5 (C$_3$), 122.0 and 123.7 (C$_{3'}$ and C$_{5'}$), 136.7 (C$_{4'}$), 149.9 (C$_{6'}$), 156.8 (C$_{2'}$). MS (*m*/*e*): 456.2 (M+H$^+$).

4.2.3. (2*RS*,3*RS*)-3-(3-Pyridyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3f). 56% Yield. Crystals, mp 121–123 °C (from methanol). ^{31}P NMR (101.2 MHz, acetone-d_6): δ 82.6. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.03 (s, 9H, Si(CH_3)$_3$), 1.41–1.51 (m, 24H, (CH_3)$_2$CHN–), 3.98–4.22 (m, 4H, (CH$_3$)$_2$CHN–), 4.58 (d, $^{3}J_{P-H}$ = 8.6 Hz, 1H, H$_3$), 7.40 (ddd, $^{3}J_{H-H}$ = 7.9 Hz, $^{3}J_{H-H}$ = 5.0 Hz, J_{H-H} = 0.5 Hz, 1H, H$_{5'}$), 7.74 (m, 1H, H$_{4'}$), 8.57 (m, 2H, H$_{2'}$ and H$_{6'}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 2.1 (Si(*C*H$_3$)$_3$), 24.4–25.5 ((*C*H$_3$)$_2$CHN–), 48.6 (d, $^{2}J_{C-P}$ = 5.7 Hz, (CH$_3$)$_2$*C*HN), 49.9 (d, $^{2}J_{C-P}$ = 4.8 Hz, (CH$_3$)$_2$*C*HN), 61.4 (C$_3$), 62.1 (d, $^{1}J_{C-P}$ = 70.6 Hz, C$_2$), 123.8 (C$_{5'}$), 132.5 (C$_{3'}$), 135.4 (C$_{4'}$), 149.3 and 150.2 (C$_{2'}$ and C$_{6'}$). MS (*m*/*e*): 456.2 (M+H$^+$).

4.2.4. (2*RS*,3*RS*)-3-(4-Pyridyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3g). 66% Yield. Crystals, mp 164–165 °C (from methanol). ^{31}P NMR (101.2 MHz, acetone-d_6): δ 27.18. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.05 (s, 9H, Si(CH_3)$_3$), 1.42–1.50 (m, 24H, (CH_3)$_2$CHN–), 3.99–4.19 (m, 4H, (CH$_3$)$_2$CHN–), 4.52 (d, $^{3}J_{P-H}$ = 8.9 Hz, 1H, H$_3$), 7.37 (m, 2H, H$_{3'}$ and H$_{5'}$), 8.60 (m, 2H, H$_{2'}$ and H$_{6'}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 2.0 (Si(*C*H$_3$)$_3$), 24.3–25.4 ((*C*H$_3$)$_2$CHN–), 48.6 (d, $^{2}J_{C-P}$ = 4.8 Hz, (CH$_3$)$_2$*C*HN), 50.0 (d, $^{2}J_{C-P}$ = 4.8 Hz, (CH$_3$)$_2$*C*HN), 62.1 (C$_3$), 62.5 (d, $^{2}J_{C-P}$ = 69.6 Hz, C$_3$), 123.0 (C$_{3'}$ and C$_{5'}$), 145.6 (C$_{4'}$), 150.4 (C$_{2'}$ and C$_{6'}$). MS (*m*/*e*): 456.2 (M+H$^+$).

4.2.5. (2*RS*,3*RS*)-3-(2-Thienyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3h). 92% Yield. Crystals, mp 134–136 °C (from methanol). ^{31}P NMR (101.2 MHz, acetone-d_6): δ 86.5. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.13 (s, 9H, Si(CH$_3$)$_3$), 1.38–1.49 (m, 24H, (CH$_3$)$_2$CHN–), 3.94-4.15 (m, 4H, (CH$_3$)$_2$CHN–), 4.66 (d, $^{3}J_{P-H}$ = 7.7 Hz, 1H, H$_3$), 7.04–7.46 (m, 2H, H$_{heterocycle}$), 7.46 (m, 1H, H$_{heterocycle}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 1.8 (Si(CH$_3$)$_3$), 24.3–25.5 ((CH$_3$)$_2$-CHN–), 48.5 (d, $^{2}J_{C-P}$ = 5.7 Hz, (CH$_3$)$_2$CHN), 49.8 (d, $^{2}J_{C-P}$ = 4.7 Hz, (CH$_3$)$_2$CHN), 60.6 (C$_3$), 63.2 (d, $^{1}J_{C-P}$ = 68.7 Hz, C$_2$), 126.4, 126.9, and 127.8 (C$_{3'}$, C$_{4'}$ and C$_{5'}$), 139.8 (C$_{2'}$). MS (*m*/*z*): 461.1 (M+H$^+$).

4.2.6. (2*RS*,3*RS*)-3-(3-Thienyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3i). 77% Yield. Crystals, mp 115–117 °C (from methanol). ^{31}P NMR (101.2 MHz, acetona-d_6): δ 82.2. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.07 (s, 9H, Si(CH_3)$_3$), 1.39–1.50 (m, 24H, (CH_3)$_2$CHN–), 3.97–4.49 (m, 4H, (CH$_3$)$_2$CHN–), 4.50 (d, $^{3}J_{P-H}$ = 8.1 Hz, 1H, H$_3$), 7.11 (d, $^{3}J_{4',5'}$ = 5.0 Hz, 1H, H$_{4'}$), 7.31 (m, 1H, H$_{2'}$), 7.51 (dd, $^{3}J_{4',5'}$ = 5.0 Hz, $^{4}J_{2',5'}$ = 3.0 Hz, 1H, H$_{5'}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 1.8 (Si(*C*H$_3$)$_3$), 24.4–25.5 ((*C*H$_3$)$_2$CHN–), 48.5 (d, $^{2}J_{C-P}$ = 4.8 Hz, (CH$_3$)$_2$*C*HN), 49.8 (d, $^{2}J_{C-P}$ = 4.8 Hz, (CH$_3$)$_2$*C*HN), 60.9 (C$_3$), 61.5 (d, $^{1}J_{C-P}$ = 70.6 Hz, C$_2$), 123.4, 127.1, and 128.0 (C$_{2'}$, C$_{4'}$ and C$_{5'}$), 138.0 (C$_{3'}$). MS (*m*/*e*): 461.1 (M+H$^+$).

4.2.7. (2*RS*,3*RS*)-3-(2-Furyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3j). 67% Yield. Crystals, mp 107–108 °C (from methanol). ^{31}P NMR (101.2 MHz, acetone-d_6): δ 82.7. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.12 (s, 9H, Si(CH_3)$_3$), 1.37–1.49 (m, 24H, (CH_3)$_2$CHN–), 3.93–4.16 (m, 4H, (CH$_3$)$_2$CHN–), 4.42 (d, $^{3}J_{P-H}$ = 7.5 Hz, 1H, H$_3$), 6.38 (m, 2H, H$_{heterocycle}$), 6.47 (m, 1H, H$_{heterocycle}$), 7.61 (m, 1H, H$_{5'}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 0.8 (Si(*C*H$_3$)$_3$), 23.8–25.0 ((*C*H$_3$)$_2$CHN-), 48.0 (d, $^{2}J_{C-P}$ = 5.5 Hz, (CH$_3$)$_2$*C*HN), 49.3 (d, $^{2}J_{C-P}$ = 5.0 Hz, (CH$_3$)$_2$*C*HN), 57.8 (C$_3$), 62.1 (d, $^{1}J_{C-P}$ = 69.6 Hz, C$_2$), 109.9 and 111.6 (C$_{3'}$ and C$_{4'}$), 143.7 (C$_{5'}$), 150.4 (C$_{2'}$). MS (*m*/*e*): 445.2 (M+H$^+$).

4.2.8. (2*RS*,3*RS*)-3-(3-Furyl)-2-trimethylsilyl-2-oxiran-2-yl-*N*,*N*,*N*′,*N*′-tetraisopropylthiophosphonodiamide (3k). 91% Yield. Crystals, mp 145–147 °C (from methanol). ^{31}P NMR (101.2 MHz, acetone-d_6): δ 82.0. ^{1}H NMR (250 MHz, acetone-d_6): δ 0.13 (s, 9H, Si(CH_3)$_3$), 1.38–1.49 (m, 24H, (CH_3)$_2$CHN–), 3.95–4.14 (m, 4H, (CH$_3$)$_2$CHN–), 4.35 (dd, $^{3}J_{P-H}$ = 8.2 Hz, $^{4}J_{H-H}$ = 1.1 Hz, 1H, H$_3$), 6.49 (dd, J_{H-H} = 1.8 Hz, J'_{H-H} = 0.8 Hz, 1H, H$_{heterocycle.}$), 7.48 (m, 1H, H$_{heterocycle}$), 7.58 (dd, $J_{H-H} = J'_{H-H}$ = 1.8 Hz, 1H, H$_{heterocycle}$). ^{13}C NMR (62.5 MHz, acetone-d_6): δ 1.9 (Si(*C*H$_3$)$_3$), 24.4–25.5 ((*C*H$_3$)$_2$CHN–), 48.4 (d, $^{2}J_{C-P}$ = 5.2 Hz, (CH$_3$)$_2$*C*HN), 49.8 (d, $^{2}J_{C-P}$ = 5.2 Hz, (CH$_3$)$_2$*C*HN), 57.8 (C$_3$), 61.5 (d,

$^{1}J_{C-P}$ = 71.0 Hz, C_2), 111.2 ($C_{4'}$), 122.0 ($C_{3'}$), 141.5 and 144.3 ($C_{2'}$ and $C_{5'}$). MS (*m/e*): 445.1 ($M+H^+$).

4.3. Crystallization and structure determination of 3d

Co-crystallization procedures were taken from the literature.[12,28] CPA and CPB solutions were added to millimolar amounts of each inhibitor, and 1 μl aliquots from the solutions were put in contact with an equal amount of reservoir solution (0.2 M magnesium acetate, 0.1 M sodium cacodylate, 0.02 M Tris–HCl (Tris: 2-amino-2-(hydroxymethyl)propane-1,3-diol), and 20% PEG 8000 (Polyethyleneglycol)). The vapor diffusion method was used, with hanging drops containing 1 μl:1 μl mixture of protein and reservoir solution. Twenty-four well Greiner plates, containing 500 μL reservoir volume and sealed with siliconized glass cover slides were left in a crystal farm at 16 °C.

Data collection of the small-molecule inhibitor **3d** was carried out on a Bruker diffractometer at the MoKα wavelength. The Wingx suite of programs[29] was used to solve, refine, and analyze the structure. This compound has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 671796.

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