Synthesis of phosphorothioate esters of L-phenyl-lactic acid as transition-state inhibitors of carboxypeptidase A

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Diastereoisomeric phosphorothioate diesters and the phosphorothioate monoester of L-phenyl-lactic acid have been prepared and their inhibitory properties with carboxypeptidase A investigated. All the phosphorothioates have K_i-values in the micromolar range, suggesting that neither the stereochemistry nor additional negative charge on the phosphorothioate moiety has a major impact on binding.

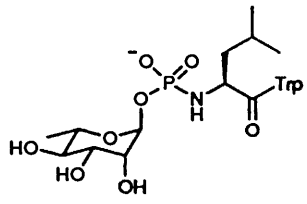
Introduction

Phosphorus(v) derivatives of amino acids and peptides have been extensively studied as inhibitors of metallo-proteases since the discovery that phosphoramidon **1** is a potent inhibitor of thermolysin.[1,2] X-Ray crystallographic analysis of the thermolysin-phosphoramidon complex established the binding interactions in the active site and led to the proposal that its phosphoramidate group, which ligates the zinc ion, resembles the putative transition state for the enzyme-catalysed reaction.[3] However, phosphoramidates are known to be labile in acidic solution.[4] We therefore chose to replace the nitrogen atom by an oxygen atom and to study phosphates rather than phosphoramidates.

Thiol-containing compounds can also be potent inhibitors of metallo-proteases. They have been extensively investigated since the discovery of captopril **2** as a potent and clinically effective inhibitor of angiotensin-converting enzyme (ACE).[5,6] This led to the development of thiol inhibitors of other metallo-proteases. 2-Benzyl-3-sulfanylpropanoic acid **3** is a very good inhibitor for carboxypeptidase A (CPA) with $K_i = 1.1 \times 10^{-8}$ mol dm^{-3}.[7] The thiol group is an effective ligand for the active-site zinc ion.

CPA hydrolyses the C-terminal residue of a peptide if it is a hydrophobic amino acid such as L-phenylalanine.[8,9] A terminal L-phenyl-lactic acid residue also satisfies this requirement. We now report the preparation of phosphorothioate benzyl esters of L-phenyl-lactic acid (compounds **8A** and **8B**) as well as the analogous phosphate ester **9**, and an investigation of their inhibitory properties with CPA. The phosphorothioate moiety is both a stable analogue of the putative transition state of the enzyme-catalysed reaction and possesses a thiolate ligand for the active-site zinc ion.

Initially, phosphorothioate diesters were prepared since the tetrahedral intermediate in hydrolysis of a peptide bond carries only one negative charge. However, Hofmann and Rottenberg have shown that a phosphate monoester of L-phenyl-lactic acid **4** carrying two negative charges on the phosphate moiety at pH 7 has a K_i-value of 0.14 μmol dm^{-3} with CPA,[10] so we have also prepared the phosphorothioate monoester of L-phenyl-lactic acid **13** and investigated its inhibition properties with CPA.



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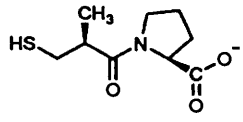

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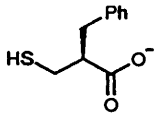

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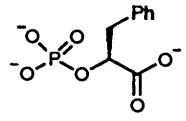

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Results and discussion

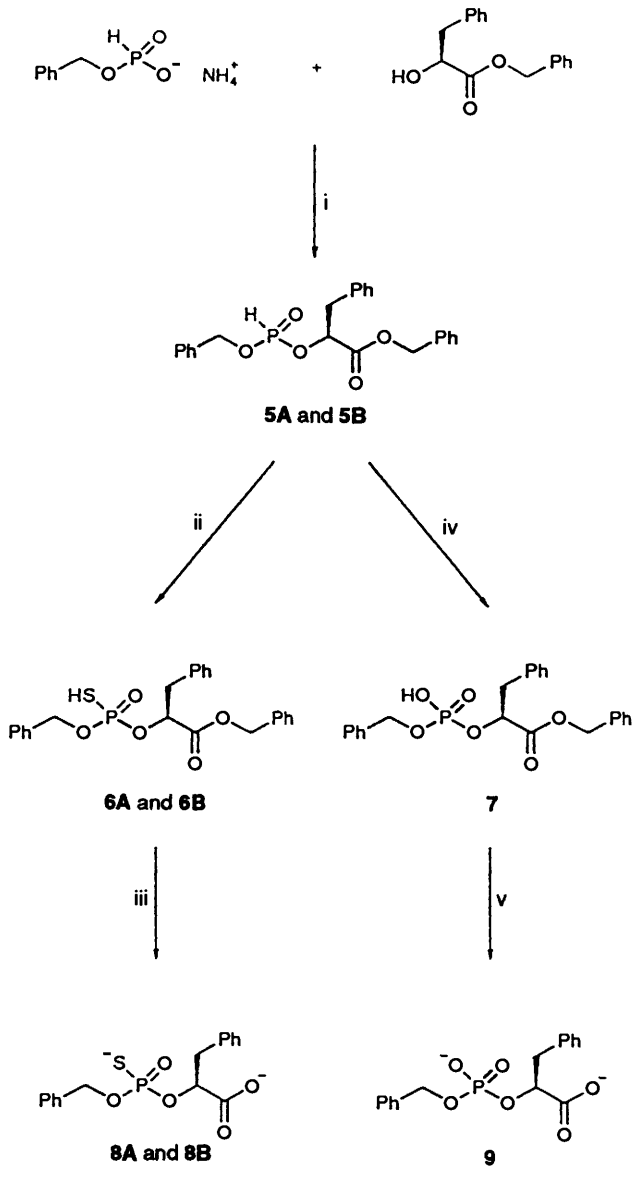
Synthesis

Preparation of the diastereoisomeric phosphorothioates diester 8A and 8B and the phosphate diester 9. The phosphorothioate benzyl esters of L-phenyl-lactic acid (compounds **8A** and **8B**) were prepared by way of the H-phosphonate esters as outlined in Scheme 1. An appealing aspect of the H-phosphonate chemistry is that both the phosphorothioate diesters **8A** and **8B** and the phosphate diester **9** can be obtained directly from the H-phosphonate intermediates **5**.

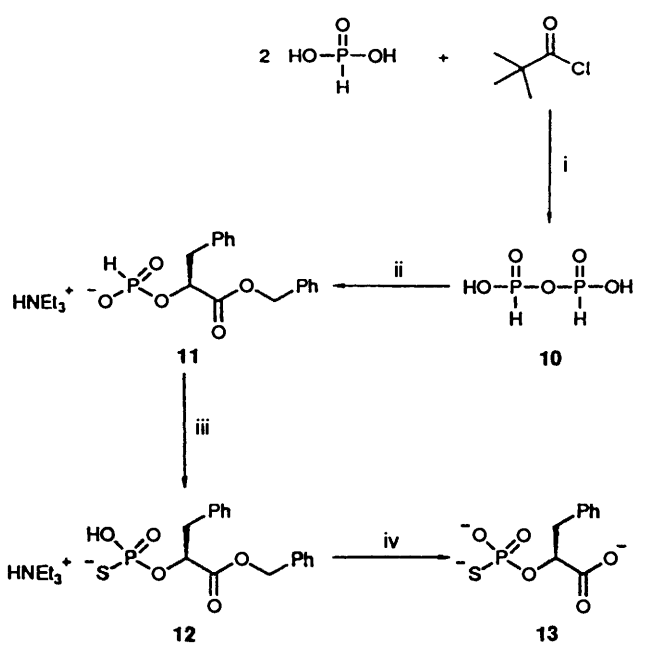
Benzyl L-phenyl-lactate was synthesized following the method of Bicknell *et al.*[11] Ammonium benzyl phosphonate was synthesized from dibenzyl phosphonate in aq. ammonia by following Hammond's procedure.[12] The condensation of ammonium benzyl H-phosphonate with benzyl L-phenyl-lactate was based on Froehler and Matteucci's method,[13] the reaction being performed at 0.1 mol dm^{-3} concentration, with 5 mol equiv. of condensing agent (pivaloyl chloride) avoiding preactivation of the H-phosphonates with coupling agent.[14] The H-phosphonates **5A** and **5B** were purified by chromatography and obtained as a clear oil in 68% yield. The two H-phosphonate diastereoisomers were separated on an analytical scale by normal-phase HPLC in sufficient quantity to be characterised by ^{1}H NMR spectroscopy.

Oxidation of the H-phosphonate diastereoisomers **5** to the phosphate diester **7** was achieved in 79% yield with an aq. iodine reagent.[15] The deprotection of the carboxylic ester with lithium hydroxide in aq. tetrahydrofuran (THF),[16] was complete in 2 h, the dilithium salt of the phosphate diester **9** being obtained quantitatively.

The sulfurisation of the H-phosphonates **5** was accomplished according to Froehler's method,[17] by treatment with a 0.1 mol dm^{-3} solution of elemental sulfur in triethylamine–carbon disulfide (1:9). The reaction was monitored by ^{31}P NMR spectroscopy and was shown to be clean and fast, being complete within 15 min. The two phosphorothioate diastereo-



Scheme 1 *Reagents*: i, Me_3CCOCl, MeCN, pyridine; ii, S_8, NEt_3, CS_2; iii, NaOH, aq. THF; iv, I_2, aq. THF–pyridine; v, LiOH, aq. THF



Scheme 2 *Reagents*: i, pyridine; ii, benzyl L-phenyl-lactate, pyridine; then TEAB; iii, Me_3SiCl, NEt_3; then S_8; iv, aq. NaOH

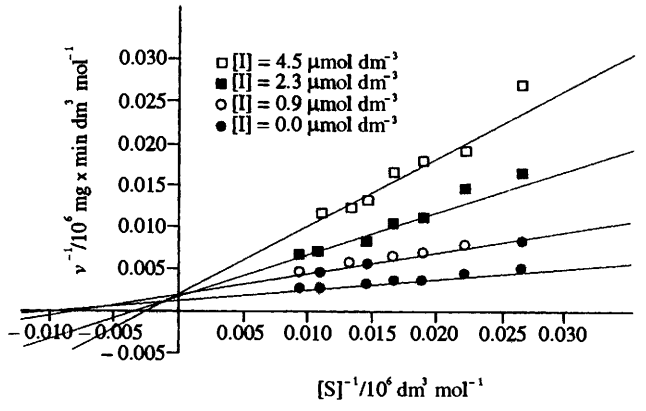


Fig. 1 Lineweaver–Burk plot of the inhibition by the thiophosphate monoester **13** of the CPA-catalysed hydrolysis of FAPP at varying substrate concentrations. The inhibitor concentration for each line is given in the figure. Conditions: 0.05 mol dm^{-3}. Tris, pH 7.5; 1 mol dm^{-3} NaCl; 25 °C.

isomers **6A** and **6B** were separated by reversed-phase HPLC, and hydrolysis of the carboxylic esters was effected on the separated phosphorothioates with sodium hydroxide in aq. THF to give the diastereoisomeric phosphorothioate diesters **8A** and **8B**.

Preparation of the phosphorothioate monoester of L-phenyl-lactic acid. The synthetic route is outlined in Scheme 2. Phosphorous acid was converted into the H-pyrophosphonate **10** with pivaloyl chloride.[18] Five mol equiv. of the H-pyrophosphonate **10** were treated with benzyl L-phenyl-lactate in pyridine and then was quenched with triethylammonium hydrogen carbonate (TEAB)[19] to give the triethylammonium *O*-(benzyl L-phenyl-lactate) H-phosphonate **11** in 96% yield. The H-phosphonate monoester was converted into trivalent phosphorus ester with trimethylsilyl chloride and this was then sulfurised *in situ* with elemental sulfur. The trimethylsilyl protecting group was removed in the work-up to give the triethylammonium phosphorothioate **12** in 87% yield. The carboxylic ester was hydrolysed to afford the trisodium salt of the *O*-phosphorothioate ester **13** with aq. sodium hydroxide in quantitative yield.

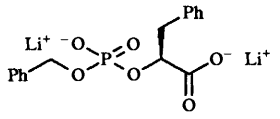
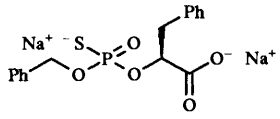
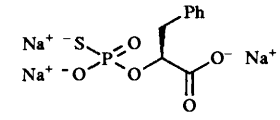
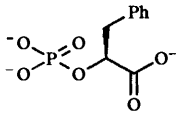
Inhibition of CPA

The enzyme-inhibition assays were performed by Hanson's procedure,[8] using the Allan form of CPA (chosen for its greater solubility)[20] and *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine (FAPP) as the substrate. The K_m-value for the substrate FAPP has been found to be 100 μmol dm^{-3}.[21] The following conditions were used: 0.05 mol dm^{-3} Tris† buffer, pH 7.5; 1 mol dm^{-3} NaCl, 25 °C. The Enzyme Kinetics programme designed by Jacek Stawinski for the Macintosh computer was used to process the data. The Lineweaver–Burk method with a fourth-power weighting algorithm and non-linear regression analysis gave consistent results (Table 1). The Lineweaver–Burk plots obtained for the inhibition of CPA by each of the inhibitors showed clearly that they behaved as competitive inhibitors. (A typical example is shown in Fig. 1.)

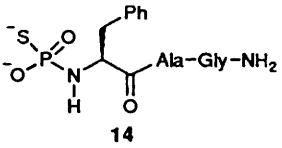
The stereochemistry of the two phosphorothioate diesters **8A** and **8B** was not determined as these compounds were found to

† Tris = 2-amino-2-(hydroxymethyl)propane-1,3-diol.

Table 1 Inhibition constants K_i of phosphate and phosphorothioate esters of L-phenyl-lactic acid with CPA (typically 8×10^{-10} mol dm^{-3}) in 0.05 mol dm^{-3} Tris buffer (pH 7.5), 1 mol dm^{-3} NaCl, 25 °C

K_i		Lineweaver–Burk (μmol dm^{-3})	Non-linear regression (μmol dm^{-3})
	**9**	0.59 ± 0.13	0.58 ± 0.12
	**8A**	1.53 ± 0.15	1.59 ± 0.12
	8B	0.60 ± 0.09	0.59 ± 0.09
	**13**	0.87 ± 0.12	0.87 ± 0.13
	**4**	0.14 [a]	

[a] The K_i for the phosphate monoester of L-phenyl-lactic acid is taken from Hofmann and Rottenberg.[10]



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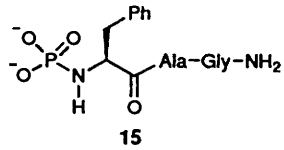

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have similar competitive inhibition constants, with K_i = 1.53 ± 0.13 μmol dm^{-3} for the thiophosphate **8A** and K_i = 0.60 ± 0.09 μmol dm^{-3} for the thiophosphate **8B** as determined by the Lineweaver–Burk method. The K_i-value for the phosphate diester **9** is 0.59 ± 0.13 μmol dm^{-3}, *i.e.* it is identical with that of the more effective of the two phosphorothioate diester inhibitors (compound **8B**) within experimental error. This leads us to conclude that, in this case at least, replacing an oxygen atom by a sulfur atom on the phosphorus does not lead to a stronger zinc ligand.

These results corroborate those obtained for the endopeptidase thermolysin by Nishino and Powers[22] who found that the thiophosphoramidate analogue **14** of a potent phosphoramidate inhibitor **15** had K_i-values of 7.0 μmol dm^{-3} and 2.6 μmol dm^{-3}, respectively. Both the results of Nishino and Powers and the results reported in Table 1 may be attributable to the fact that a P–S bond, at 1.85 Å, is considerably longer than a P–O bond, at 1.39 Å.[23] This could cause steric congestion around the zinc ion of the metallo-protease and lead to a weakening of some of the enzyme–inhibitor interactions.

Nishino and Powers suggested that the thiophosphoryl peptide might be ligating to the zinc ion through one of its oxygen atoms instead of sulfur and that the difference in K_i-values could then be due to the differences of electron density on the oxygen atoms of the two functional groups.[22] Since we synthesized both diastereoisomers of the phosphorothioate diester (compounds **8A** and **8B**), one of them at least would be expected to be able to bind in such a way that it could use the sulfur as a zinc-ion ligand. Since the single charge on the phosphorothioate group will be localised on the sulfur,[24] it seems likely that both diastereoisomers use the thiolate ion as the ligand to the active-site zinc ion but the additional ligation ability of the thiolate ion for the zinc ion is offset by the difficulty in accommodating the long P–S$^-$ bond.

The phosphorothioate monoester **13** was found to have an inhibition constant K_i equal to 0.87 ± 0.12 μmol dm^{-3}. This value is quite similar to those for the diastereoisomeric phosphorothioate diesters **8A** and **8B** and shows that adding a second charge to the thiophosphoryl group does not enhance the activity of the inhibitor. As in the case of the two phosphorothioate diesters **8A** and **8B**, a possible explanation of this result is that the P–S bond is longer than the P–O bond and the enzyme–inhibitor interactions may thus be less good for the phosphorothioate **13** than for the phosphate monoester of L-phenyl-lactic acid.

It should also be noted that in the case of the phosphorothioate diesters **8A** and **8B** and of the phosphorothioate **13**, the sulfur atom is one bond further away from the carboxy group than in the case of the more potent inhibitor **3**.

Experimental

The Allan form of CPA (EC 3.4.17.1) was used because of its greater solubility. The assays of the inhibitors were performed according to Hanson's procedure,[8] under the following conditions: 25 °C; 0.05 mol dm^{-3} Tris, pH 7.5; 1.0 mol dm^{-3} NaCl. CPA assay solutions were freshly prepared and bovine serum albumin was added to stabilise the CPA.[8] FAPP was the substrate used for the assays. Deionised water, purified with an Elga Maxima system, was used for the preparation of all assay solutions. CPA and FAPP were purchased from Sigma Chemical Company Ltd, Poole, England.

The kinetic data for the inhibitors was determined as follows. Generally, four separate series of runs were done for each inhibitor and for each of these series, four different inhibitor concentrations were run against six different substrate concentrations with duplicates.[25]

Phosphate **9**: the inhibitor concentrations range from 0.0 to 4.6 μmol dm^{-3} and the substrate concentrations range from 32.1 to 112.2 μmol dm^{-3}. The resulting initial velocities measured range from 0.006 to 0.095 min^{-1}.

Phosphorothioate diester **8A**: the inhibitor concentrations range from 0.0 to 11.1 μmol dm^{-3} and the substrate concentrations range from 39.5 to 170.5 μmol dm^{-3}. The resulting initial velocities measured range from 0.007 to 0.066 min^{-1}.

Phosphorothioate diester **8B**: the inhibitor concentrations range from 0.0 to 2.3 μmol dm^{-3} and the substrate concentrations range from 36.7 to 151.1 μmol dm^{-3}. The resulting initial velocities measured range from 0.010 to 0.065 min^{-1}.

Phosphorothioate monoester **13**: the inhibitor concentrations range from 0.0 to 4.5 μmol dm^{-3} and the substrate concentrations range from 16.5 to 105.8 μmol dm^{-3}. The resulting initial velocities measured range from 0.004 to 0.106 min^{-1}.

Mps were recorded on a Kofler block apparatus and are quoted uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter, and are given in units of 10^{-1} deg cm^2 g^{-1}, IR spectra were recorded on a Perkin-Elmer 1750 Infrared Fourier Transform spectrometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AM-500 (500 MHz) spectrometer and referenced internally to residual $CHCl_3$ (δ_H 7.27) in $CDCl_3$, to the residual solvent peak (δ_H 2.502) in $(CD_3)_2SO$ ([$^2H_6$]DMSO) and to 1,4-dioxane (δ_H 3.75) in D_2O. The HOD signal in the ^{1}H NMR spectra of aqueous solutions was suppressed. *J*-Values are in Hz. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AM 250 (62.9 MHz) spectrometer and on a Bruker AMX 500 (125.7 MHz) spectrometer and referenced internally to $CDCl_3$ (δ_C 77.0), [$^2H_4$]MeOH (δ_C 49.0) or 1,4-dioxane (δ_C 67.3) for aqueous solutions. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra were recorded on a Bruker AM 250 (101.7 MHz). The solution to be measured was in an 8 mm tube which was placed inside a 10 mm tube containing D_2O as lock solvent and orthophosphoric acid (δ_P 0.0) as reference. Mass spectra were recorded on a VG 20-250 masslab spectrometer for desorption chemical ionisation (DCI, NH_3) and a VG BIOQ Biotech for negative-ion electrospray. TLC was performed on Merck D.C.-Alufolien Kieselgel 60 F_{254} 0.2 mm pre-coated plates. Spot detection was by UV light fluorescence, iodine vapour or acidic palladium chloride solution in methanol. Flash chromatography was carried out on Janssen Chimica C60 silicagel and on Sorbsil C60 40/60 H synthetic amorphous silica. Ion-exchange chromatography was carried out using DEAE Sephadex A25. High-performance liquid chromatography (HPLC) was preformed on a system comprising of a Waters 600E System Controller and a Waters 990 photodiode array detector. HPLC columns used for normal-phase separation included a Hypersil silica analytical column (5μ particle size; OD: 1/4 in × 250 mm) and a Hypersil silica semi-preparative column (5μ particle size; OD: 1/2 in × 250 mm). HPLC columns used for reversed phase separations included a Hypersil reversed phase silica analytical column (5μ particle size; OD: 1/4 in × 250 mm) and a Zorbax C18 reversed phase silica preparative column (7μ particle size; 21.2 × 250 mm). Chemicals were obtained from Aldrich Chemical Company Ltd., Gillingham, Dorset, England, Avocado Research Chemicals Ltd., Lancaster, Lancashire, England, Fluka Chemika-Biochemika, Glossop, Derbyshire, England and Lancaster Synthesis Ltd., Morecambe, Lancashire, England. Distilled water was used in chemical experiments. Solvents and standard laboratory reagents were purified by traditional methods[26] where necessary.

Synthesis of *O*-benzyl *O'*-(benzyl L-phenyl-lactate) H-phosphonate 5 (two diastereoisomers)

To a solution of benzyl L-phenyl-lactate[11] (5.596 g, 0.022 mol) and ammonium benzyl phosphonate[12] (4.111 g, 0.022 mol) in anhydrous pyridine–anhydrous acetonitrile (1:1, v/v; 200 cm^3) was added pivaloyl chloride (14 cm^3, 5.2 mol equiv.).[27,28] The reaction mixture was stirred overnight at room temp. The solvent was evaporated off under reduced pressure and the residue was dissolved in dichloromethane and the solution was washed with 0.5 mol dm^{-3} H_2SO_4. The organic layer was dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give a clear oil. The sample was purified by flash chromatography, with ethyl acetate–light petroleum (distillation range 40–60 °C) (1:2, v/v) as eluent (R_f 0.25). The *product* was collected as a clear oil (6.064 g, 68%) as a 48:52 mixture of diastereoisomers (Found: C, 67.1; H, 5.4. $C_{23}H_{23}O_5P$ requires C, 67.31; H, 5.65%); ν_{max}(liq. film)/cm^{-1} 1752 (CO) and 2455 (PH); δ_C(125.7 MHz; $CDCl_3$) 169.6 (CO), 169.3 (CO), 135.4 (Ph C), 135.2 (Ph C), 134.7 (Ph C), 129.4 (Ph CH), 128.6 (Ph CH), 128.5 (Ph CH), 128.3 (Ph CH), 127.9 (Ph CH), 127.7 (Ph CH), 127.2 (Ph CH), 127.1 (Ph CH), 126.8 (Ph CH), 75.2 (CH, d, $^2J_{PC}$ 6.3), 75.1 (CH), 67.7 (CO_2CH_2Ph), 67.5 (CO_2CH_2Ph), 66.8 ($POCH_2Ph$, d, $^2J_{PC}$ 5.6), 66.0 ($POCH_2Ph$, d, $^2J_{PC}$ 5.6), 39.0 ($CHCH_2Ph$) and 38.5 ($CHCH_2Ph$, d, $^3J_{PC}$ 5.7); $\delta_P\{^1H\}$ (101.26 MHz; $CHCl_3$) 7.8 and 6.9; *m/z* (DCI, NH_3) 411 (MH^+, 13%), 274 (benzyl L-phenyl-lactate + NH_4^+, 68), 228 (23), 192 (15), 108 ($PhCH_2OH$) and 91 ($PhCH_2^+$, 100).

The two H-phosphonate diastereoisomers were separated by normal-phase HPLC using a Hypersil silica semi-preparative column. The HPLC column was eluted with ethyl acetate–hexane (28:72). The flow rate was 4 cm^3 min^{-1}. The separation was monitored by UV spectroscopy at a wavelength of 254 nm. A number of 2 mm^3 (1.20 mol dm^{-3}) injections were necessary in order to obtain sufficient material for a ^{1}H NMR spectrum. The H-phosphonate **5A** (less polar) was collected at a retention time (t_R) of 15.5 min and the H-phosphonate **5B** (more polar) was collected at t_R 16.5 min.

H-phosphonate 5A (less polar). δ_H(500 MHz; $CDCl_3$) 7.39–7.15 (15 H, m, 3 Ph), 6.63 (1 H, d, $^1J_{PH}$ 727.9, PH), 5.21 (1 H, d, J_{AB} 12.2, CO_2CHHPh), 5.17 (1 H, d, J_{AB} 12.2, CO_2CHHPh), 5.16 (1 H, ddd, $^3J_{PH}$ ~ 14, J_{AX} 4.3, J_{BX} 9.0, $CHCH_2$), 5.05 (1 H, dd, $^3J_{PH}$ 8.6, J_{AB} 11.8, $POCHHPh$), 4.97 (1 H, dd, $^3J_{PH}$ 8.5, J_{AB} 11.8, $POCHHPh$), 3.29 (1 H, ddd, $^4J_{PH}$ 1.6, J_{AB} 14.3, J_{AX} 4.3, $CHCHHPh$) and 3.12 (1 H, dd, J_{AB} 14.3, J_{BX} 9.0, $CHCHHPh$).

H-phosphonate 5B (more polar). δ_H(500 MHz; $CDCl_3$) 7.42–7.15 (15 H, m, 3 Ph), 7.04 (1 H, d, $^1J_{PH}$ 735.9, PH), 5.24 (1 H, d, J_{AB} 12.1, CO_2CHHPh), 5.22 (1 H, ddd, $^3J_{PH}$ 13.2, J_{AX} 4.0, J_{BX} 8.9, $CHCH_2$), 5.19 (1 H, d, J_{AB} 12.1, CO_2CHHPh), 4.81 (1 H, dd, $^3J_{PH}$ 8.8, J_{AB} 11.8, $POCHHPh$), 4.60 (1 H, dd, $^3J_{PH}$ 8.2, J_{AB} 11.8, $POCHHPh$), 3.28 (1 H, ddd, $^4J_{PH}$ 2.1, J_{AB} 14.3, J_{AX} 4.0, $CHCHHPh$) and 3.08 (1 H, dd, J_{AB} 14.3, J_{BX} 8.9, $CHCHHPh$).

Synthesis of *O*-benzyl *O'*-(benzyl L-phenyl-lactate) hydrogen phosphate 7

The two diastereoisomers of *O*-benzyl *O'*-(benzyl L-phenyl-lactate) H-phosphonate **5** (0.500 g, 1.22 mmol) were dissolved in DNA–RNA-oxidising solution from Cruachem (16 cm^3; 0.1 mol dm^{-3} iodine in THF–pyridine–water, 1.3 mol equiv.). The reaction mixture was stirred at room temp. for 15 min; the appearance of a precipitate (pyridine hydroiodide) was observed within 5 min. Aq. $Na_2S_2O_3 \cdot H_2O$ (0.400 g, 1.61 mmol in 3 cm^3) was added to destroy the excess of iodine. An immediate loss of colour of the solution was observed. The reaction mixture was stirred for 20 min. The solvent was then evaporated off under reduced pressure. The residue was partitioned between ethyl acetate and water, and the organic layer was separated and dried ($MgSO_4$), filtered, and evaporated under reduced pressure. This yielded an orange oil (0.411 g, 79%), $[\alpha]_D^{25}$ −21.3 (*c* 0.792, $CHCl_3$); ν_{max}(liq. film)/cm^{-1}

1752 (CO); δ_H(500 MHz; $CDCl_3$)‡ 7.41–7.06 (15 H, m, 3 × Ph), 5.04 (1 H, d, J_{AB} 12.2, CO_2CHHPh), ~5 (1 H, ddd, J_{AX} 5.8, J_{BX} 6.6, $^3J_{PH}$ 14.2, CH), 4.99 (1 H, d, J_{AB} 12.2, CO_2CHHPh), 4.83 (1 H, dd, $^3J_{PH}$ 6.7, J_{AB} 12.0, $POCHHPh$), 4.76 (1 H, dd, $^3J_{PH}$ 6.5, J_{AB} 12.0, $POCHHPh$), 3.12 (1 H, dd, J_{AB} 14.1, J_{AX} 5.8, $CHCHHPh$) and 3.07 (1 H, dd, J_{AB} 14.1, J_{BX} 6.6, $CHCHHPh$); δ_C(125.7 MHz; $CDCl_3$) 170.4 (CO), 137.0 (d, $^3J_{PC}$ 8.4, $POCH_2Ph$, *ipso* C), 135.6 (Ph C), 135.2 (Ph C), 129.7 (Ph CH), 128.6 (Ph CH), 128.4 (Ph CH), 128.4 (Ph CH), 128.2 (Ph CH), 127.6 (Ph CH), 127.5 (Ph CH), 126.8 (Ph CH), 75.3 (CH), 68.0 ($^2J_{PC}$ 3.9, $POCH_2Ph$, d), 66.8 (CO_2CH_2Ph) and 39.3 ($^3J_{PC}$ 5.7, $CHCH_2Ph$, d); δ_P {^{1}H} (101.26 MHz; $CHCl_3$) −2.8; *m/z* (negative-ion electrospray) 425 [(M − H)$^-$, 100%] and 426 (26).

Synthesis of dilithium *O*-benzyl *O'*-(L-phenyl-lactate) phosphate 9

O-Benzyl *O'*-(benzyl L-phenyl-lactate) hydrogen phosphate **7** (0.162 g, 0.380 mmol) and $LiOH{\cdot}H_2O$ (0.034 g, 2.1 mol equiv.) were dissolved in THF–water (3:1, v/v; 3 cm^3 ml) at 10 °C. The reaction mixture was stirred for 2 h at 10 °C. The solvent was then removed under reduced pressure and the residue was dissolved in water and the solution was washed with dichloromethane. The organic phase showed the presence of benzyl alcohol but no trace of starting material. The aqueous phase was concentrated under reduced pressure and freeze dried. A solid (0.134 g, quantitative) was obtained, mp > 230 °C; $[\alpha]_D^{25}$ −3.8 (*c* 0.546, water); ν_{max}(KBr disc)/cm^{-1} 1592 (CO); δ_P {^{1}H} (101.26 MHz; water) −0.8; *m/z* (negative-ion electrospray) 341 [(M − Li) −21%], 335 [(M − 2Li + H)$^-$, 34], 227 [(M − 2Li + H − OCH_2Ph)$^-$, 23] and 186.9 ($PhCH_2OPO_3H^-$, 100).

The compound was further purified by ion-exchange chromatography with TEAB (pH 7.8; 0.05–0.5 mol dm^{-3}) as eluent to give the bis(triethylammonium)*O*-benzyl *O'*-(L-phenyl-lactate) phosphate **9**, δ_H(500 MHz; D_2O)§ 7.44–7.28 (10 H, m, 2 × Ph), ~4.8 (1 H, ddd, J_{AX} 5.3, J_{BX} 7.0, CH), 4.70 (1 H, dd, $^3J_{PH}$ 7.4, J_{AB} 12.1, $POCHHPh$), 4.50 (1 H, dd, $^3J_{PH}$ 6.5, J_{AB} 12.1, $POCHHPh$), 3.19 [6 H, q, *J* 7.3, $(MeCH_2)_3NH^+$], 3.12 (1 H, dd, J_{AB} 14.0, J_{AX} 5.3, CO_2CHHPh), 3.04 (1 H, dd, J_{AB} 14.0, J_{BX} 7.0, CO_2CHHPh) and 1.27 [9 H, t, *J* 7.3 $(MeCH_2)_3NH^+$]; δ_C(125.7 MHz; [$^2H_4$]^2 MeOH) 175.9 (CO), 139.7 ($^3J_{PC}$ 8.2, Ph C, d), 138.6 (Ph C), 131.0 (Ph CH), 129.2 (Ph CH), 129.2 (Ph CH), 128.5 (Ph CH), 128.3 (Ph CH), 127.6 (Ph CH), 77.0 (CH), 68.0 ($POCH_2Ph$), 43.0 [2 × $(MeCH_2)_3NH^+$], 43.0 ($CHCH_2Ph$) and 9.1 [2 × $(MeCH_2)_3NH^+$].

Synthesis of triethylammonium *O*-benzyl *O'*-(benzyl L-phenyl-lactate) *S*-hydrogen phosphorothioates 6A and 6B

The conversion of the H-phosphonate diesters **5** into the thiophosphate analogues was performed by following Froehler's method.[17] The H-phosphonates **5** (0.763 g, 1.859 mmol) and elemental sulfur (0.060 g, 1.871 mmol) were dissolved in triethylamine–carbon disulfide (1:9, v/v; 20 cm^3). The reaction mixture was stirred for 15 min at room temp. It was then evaporated under reduced pressure to yield the pure *product* (1.010 g, quantitative) (Found: C, 63.9; H, 7.2; N, 2.2. $C_{29}H_{38}NO_5PS$ requires C, 64.07; H, 7.05; N, 2.58%); ν_{max}(liq. film)/cm^{-1} 1752 (CO); δ_P {^{1}H} (101.26 MHz; $CHCl_3$) 57.9 and 57.8; *m/z* (negative-ion electrospray) 444 (2%), 443 (9), 442 (32) and 441 (M^-, 100).

The two phosphorothioate diastereoisomers **6A** and **6B** were separated by reversed-phase HPLC using a preparative column. The HPLC column was eluted with methanol–aq. TEAB (0.05 mol dm^{-3}, pH 8) (65:35, v/v) at a flow rate of 4 cm^{-3} min^{-1}. A number of 70 mm^3 (0.056 mol dm^{-3}) injections were executed. The more polar phosphorothioate diastereoisomer **6A** was collected at t_R 61 min, and the less polar phosphorothioate diastereoisomer **6B** at t_R 73 min.

Phosphorothioate 6A (more polar). δ_H(500 MHz; $CDCl_3$)¶ 11.9 (1 H, br s, NH), 7.31–7.13 (15 H, m, 3 × Ph), 5.17 (1 H, ddd, $^3J_{PH}$ 11.6, J_{AX} 6.2, J_{BX} 6.5, CH), 4.99 (1 H, d, J_{AB} 12.3, CO_2CHHPh), 4.97 (1 H, d, J_{AB} 12.3, CO_2CHHPh), 4.96 (1 H, dd, J_{AB} 12.2, $^3J_{PH}$ 8.2, $POCHHPh$), 4.79 (1 H, dd, J_{AB} 12.2, $^3J_{PH}$ 7.8, $POCHHPh$), 3.19 (1 H, dd, J_{AB} 13.8, J_{AX} 6.2, $CHCHHPh$), 3.17 (1 H, dd, J_{AB} 13.8, J_{BX} 6.5, $CHCHHPh$), 2.93 [6 H, dq, *J* 7.3, J_{NHCH} 4.4, $(MeCH_2)_3NH^+$] and 1.18 [9 H, t, *J* 7.3, $(MeCH_2)_3NH^+$]; δ_C(62.9 MHz; $CDCl_3$) 171.1 (d, $^3J_{PC}$ 3.4, C=O), 138.4 (d, $^3J_{PC}$ 9.1, *ipso*-C, $POCH_2Ph$), 136.3 (Ph C), 135.5 (Ph C), 129.7 (Ph CH), 128.8 (Ph CH), 128.2 (Ph CH), 128.1 (Ph CH), 128.0 (Ph CH), 127.9 (Ph CH), 127.5 (Ph CH), 127.1 (Ph CH), 126.4 (Ph CH), 75.5 (d, $^2J_{PC}$ 5.2, CH), 67.7 (d, $^2J_{PC}$ 5.0, $POCH_2Ph$), 66.2 (CO_2CH_2Ph), 45.4 [$MeCH_2)_3NH^+$], 39.6 (d, $^3J_{PC}$ 5.3, $CHCH_2Ph$) and 8.4 [$(MeCH_2)_3NH^+$].

Phosphorothioate 6B (less polar). δ_H(500 MHz; $CDCl_3$)‖ 7.42–7.16 (15 H, m, 3 × Ph), 5.24 (1 H, ddd, $^3J_{PH}$ 11.5, J_{AX} 6, J_{BX} 6, CH), 5.09 (1 H, d, J_{AB} 12.4, CO_2CHHPh), 5.06 (1 H, d, J_{AB} 12.4, CO_2CHHPh), 4.86 (1 H, dd, J_{AB} 12.2, $^3J_{PH}$ 8.3, $POCHHPh$), 4.75 (1 H, dd, J_{AB} 12.2, $^3J_{PH}$ 7.9, $POCHHPh$), 3.12 (1 H, dd, J_{AB} 14, J_{AX} 6, $CHCHHPh$), 3.11 (1 H, dd, J_{AB} 14, J_{BX} 6, $CHCHHPh$), 2.97 [6 H, dq, *J* 7.3 and 0.8, $(MeCH_2)_3NH^+$] and 1.20 [9 H, t, *J* 7.3, $(MeCH_2)_3NH^+$]; δ_C(62.9 MHz; $CDCl_3$) 171.4 (d, $^3J_{PC}$ 5.6, C=O), 138.5 (d, $^3J_{PC}$ 9.2, *ipso*-C, $POCH_2Ph$), 136.5 (Ph C), 135.6 (Ph C), 129.8 (Ph CH), 128.3 (Ph CH), 128.2 (Ph CH), 128.1 (Ph CH), 128.0 (Ph CH), 127.5 (Ph CH), 127.2 (Ph CH), 126.5 (Ph CH), 75.5 (d, $^2J_{PC}$ 5.5, CH), 67.5 (d, $^2J_{PC}$ 5.5, $POCH_2Ph$), 66.3 (CO_2CH_2Ph), 45.4 [$(MeCH_2)_3NH^+$], 39.4 (d, $^3J_{PC}$ 5.5, $CHCH_2Ph$) and 8.4 [$(MeCH_2)_3NH^+$].

Synthesis of disodium *O*-benzyl *O'*-(L-phenyl-lactate) phosphorothioate 8A and 8B

The phosphorothioates **6A** and **6B** (0.670 g, 1.23 mmol) as a solution in THF (7.5 cm^3) were treated with 1 mol dm^{-3} NaOH (2.6 cm^3; 2.11 mol equiv.) at room temp. for 30 min. The reaction mixture was then evaporated under reduced pressure, THF (7.5 cm^3) was added and the reaction mixture was stirred at room temp. for 30 min. This procedure was repeated once in order to eliminate the triethylamine and to facilitate complete exchange of the counter-ion. The reaction mixture was then evaporated under reduced pressure, the residue was dissolved in water, and the solution was washed with dichloromethane. The organic phase showed the presence of benzyl alcohol but no trace of starting material. The aqueous layer was concentrated under reduced pressure and freeze dried. A yellow solid (0.448 g, 92%) was obtained, mp > 230 °C; ν_{max}(KBr disc)/cm^{-1} 1598 (CO); δ_P {^{1}H} (101.26 MHz; water) 54.5 and 54.3; *m/z* (negative-ion electrospray) 353 (9%), 352 (21), 351 (MH^-, 100), 336 (4) and 335 [(MH-O)$^-$, 19].

The reaction was also performed separately under the same

‡ This spectrum was complicated by the fact that the CH peaks were somewhat obscured by the CO_2CH_2Ph peaks. The precise shift of the CH peak could not be determined and the $^3J_{PH}$ coupling constant for the CH was determined from a spectrum obtained in [$^2H_6$]benzene at 500 MHz.

§ The CH peaks were slightly obscured by the HOD peak therefore it was impossible to determine the shift precisely; it was also impossible to determine the $^3J_{PH}$ coupling constant between the CH proton and the phosphorus.

¶ The $^3J_{PH}$ coupling constant for the CH was obtained by a selective decoupling experiment. The $CHCH_2Ph$ peaks (δ_H 3.18) were irradiated and the CH peak (δ_H 5.17) was observed. It was thus simplified to a dd.

‖ The $^3J_{PH}$ coupling constant for the CH was obtained by a selective decoupling experiment. The $CHCH_2Ph$ peaks (δ_H 3.12) were irradiated and the CH peak (δ_H 5.24) was observed. It was thus simplified to a dd.

conditions on the separated phosphorothioates **6A** and **6B** to give:

Phosphorothioate diester 8A. δ_H(500 MHz; [$^2H_6$] DMSO) 7.30–7.08 (10 H, m, 2 Ph), 4.69 (1 H, dd, J_{AB} 12.6, $^3J_{PH}$ 7.7, POC*H*HPh), 4.48 (1 H, ddd, $^3J_{PH}$ 10.3, J_{AX} 5.4 J_{BX} 6.5, CH), 4.22 (1 H, dd, J_{AB} 12.6, $^3J_{PH}$ 6.2, POCH*H*Ph), 3.03 (1 H, dd, J_{AB} 13.6, J_{AX} 5.4, CHC*H*HPh) and 2.90 (1 H, dd, J_{AB} 13.6, J_{BX} 6.5, CHCH*H*Ph).

Phosphorothioate diester 8B. δ_H(500 MHz; [$^2H_6$]DMSO) 7.31–7.08 (10 H, m, 2 × Ph), 4.84 (1 H, ddd, $^3J_{PH}$ 11.1, J_{AX} 4.1, J_{BX} 8.2, CH), 4.46 (1 H, dd, J_{AB} 12.8, $^3J_{PH}$ 7.8, POC*H*HPh), 3.97 (1 H, dd, J_{AB} 12.8, $^3J_{PH}$ 5.9, POCH*H*Ph), 3.10 (1 H, ddd, J_{AB} 14.0, J_{AX} 4.1, $^4J_{PH}$ ~ 2.5, CHC*H*HPh) and 2.90 (1 H, dd, J_{AB} 14.0, J_{BX} 8.2, CHCH*H*Ph); δ_C(125.7 MHz; D_2O) 178.9 (C=O), 138.3 (Ph C), 130.6 (Ph CH), 129.3 (Ph CH), 129.1 (Ph CH), 128.7 (Ph CH), 128.3 (Ph CH), 127.4 (Ph CH), 78.9 (d, $^2J_{PC}$ 5.7, CH), 68.0 (d, $^2J_{PC}$ 5.4, PO*C*H_2Ph) and 40.3 (CH*C*H_2Ph).

Synthesis of triethylammonium *O*-(benzyl L-phenyl-lactate) H-phosphonate 11

The H-phosphonate **11** was prepared by following the method of Stawinski and Thelin.[18,19] Phosphorous acid (3.397 g, 41.42 mmol) was dissolved in anhydrous pyridine (20 cm^3), $\delta_P\{^1H\}$ (101.26 MHz; pyridine) 1.7. Pivaloyl chloride (2.8 cm^3, 22.7 mmol) was added to form the pyrophosphonate in an exothermic reaction, $\delta_P\{^1H\}$ (101.26 MHz; pyridine) −7.0. Benzyl L-phenyl-lactate (0.870 g, 3.39 mmol) was added and the reaction mixture was stirred at room temp. for 6 h before being quenched with mol dm^{-3} aq. TEAB (pH 7.5; 20 cm^3). It was then evaporated under reduced pressure the residue was dissolved in dichloromethane (150 cm^3), and the solution was washed with 0.5 mol dm^{-3} TEAB (pH 7.25; 3 × 50 cm^3). The organic layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure. This yielded the desired product **11** as a clear, slightly yellow oil (1.371 g, 96%), $[\alpha]_D^{25}$ −19.8 (*c* 0.70, $CHCl_3$); ν_{max}(liq. film, NaCl plates)/cm^{-1} 1737 (CO); δ_H(500 MHz; $CDCl_3$) 12.38 (1 H, br s, Et_3NH^+), 7.34–7.13 (10 H, m, 2 × Ph), 6.80 (1 H, d, $^1J_{PH}$ 633.3, PH), 5.10 (1 H, d, J_{AB} 12.3, CO_2CHHPh), 5.08 (1 H, d, J_{AB} 12.3, CO_2CHHPh), 5.05 (1 H, ddd, $^3J_{PH}$ 9.7, J_{AX} 5.3, J_{BX} 7.2, CH), 3.16 (1 H, dd, J_{AB} 14.0, J_{AX} 5.3, CHC*H*HPh), 3.09 (1 H, dd, J_{AB} 14.0, J_{BX} 7.2, CHCH*H*Ph), 2.88 [6 H, q, *J* 7.3, Me*C*H_2)$_3$NH$^+$] and 1.17 [9 H, t, *J* 7.3, *Me*CH$_2$)$_3$NH$^+$]; δ_C(125.7 MHz; $CDCl_3$) 171.3 (CO), 136.5 (Ph C), 136.2 (Ph C), 129.6 (Ph CH), 128.3 (Ph CH), 128.1 (Ph CH), 128.0 (Ph CH), 126.4 (Ph CH), 73.2 (CH), 66.4 (CO_2CH_2Ph), 45.1 [Me*C*H$_2$)$_3$NH$^+$], 39.6 (CH*C*H$_2$Ph) and 8.3 [(*Me*CH$_2$)$_3$NH$^+$]; δ_P $\{^1H\}$ (101.26 MHz; pyridine) 2.7; *m*/*z* (negative-ion electrospray) 320 (19%), 319 (M^-, 100), 228.9 [(M − PhCH$_2$)$^-$, 11] and 210.8 [(M − PhCH$_2$OH)$^-$, 15].

Synthesis of triethylammonium *O*-(benzyl L-phenyl-lactate)hydrogen phosphorothioate 12

The phosphorothioate **12** was synthesized by following the method of Hata and Sekine.[29] A solution of the H-phosphonate monoester **11** (0.830 g, 1.969 mmol) in dry pyridine (20 cm^3) was treated with triethylamine (1.40 cm^3, 5 mol equiv.) and trimethylsilyl chloride (1.25 cm^3, 5 mol equiv.) at room temp. Immediate formation of a precipitate was observed. Elemental sulfur was then added (0.126 g, 2 mol equiv.) and the reaction mixture was stirred at room temp. for 1 h, $\delta_P\{^1H\}$ (101.26 MHz; pyridine) 43.6. Water was then added and the reaction mixture was evaporated under reduced pressure. As the product was slightly water soluble, the residue was dissolved in water and this solution was filtered to remove the excess of sulfur. After removal of the solvent, the residue was dissolved in dichloromethane (150 cm^3) and the solution was washed with water (3 × 50 cm^3). The organic phase was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The desired product **12** was obtained as a slightly yellow oil (0.779 g, 87%), $[\alpha]_D^{25}$ −12.3 (*c* 0.56, $CHCl_3$); ν_{max}(liq. film, NaCl plates)/cm^{-1} 1752 (CO); δ_H(500 MHz; $CDCl_3$) 7.38–7.14 (10 H, m, 2 × Ph), 5.33 (1 H, dt, $^3J_{PH}$ 12.0, *J* 6.0, CH), 5.08 (1 H, d, J_{AB} 12.4, CO_2CHHPh), 5.07 (1 H, d, J_{AB} 12.4, CO_2CHHPh), ~4.4 (2 H, br s, SH and NH$^+$), 3.23 (2 H, d, *J* 6.0, CHCH_2Ph), 3.02 [6 H, q, *J* 7.3, (MeCH_2)$_3$NH$^+$] and 1.22 [9 H, t, *J* 7.3, (MeCH$_2$)$_3$NH$^+$]; δ_C(125.7 MHz; $CDCl_3$) 170.9 (CO, d, $^3J_{PC}$ 5.4, 136.0 (Ph C), 135.2 (Ph C), 129.6 (Ph CH), 128.0 (Ph CH), 127.9 (Ph CH), 127.8 (Ph CH), 127.7 (Ph CH), 126.1 (Ph CH), 74.7 (CH), 66.0 (CO_2CH_2Ph), 45.4 [(Me*C*H$_2$)$_3$NH$^+$], 39.1 (CH*C*H$_2$Ph) and 8.3 [(MeCH$_2$)$_3$NH$^+$]; δ_P(101.26 MHz; $CHCl_3$) 55.5 ($^3J_{PH}$ 12.0 Hz); *m*/*z* (negative-ion electrospray) 351 [(M − H), 100%], 352(21) and 353(8).

Synthesis of trisodium *O*-(L-phenyl-lactate) phosphorothioate 13

The phosphorothioate **12** (0.419 g, 0.972 mmol) was stirred with 1 mol dm^{-3} NaOH (3 cm^3, 3.1 mol equiv.) and THF (6 cm^3) at room temp. 1.5 h. The reaction mixture was then evaporated under reduced pressure, the residue was dissolved in water (50 cm^3), and the solution was washed with dichloromethane (3 × 15 cm^3). The organic phase showed the presence of benzyl alcohol but no trace of starting material (by ^1H NMR spectroscopy). The aqueous layer was concentrated under reduced pressure and freeze dried. This yielded the product **13** as a solid (0.324 g, quantitative), mp > 230 °C; $[\alpha]_D^{25}$ +25.1 (*c* 0.578, water); ν_{max}(KBr disc)/cm^{-1} 1598 (CO); δ_H(500 MHz; [$^2H_4$]MeOH) 7.51–7.10 (5 H, m, Ph), 4.88 (1 H, ddd, $^3J_{PH}$ 10.9, J_{AX} 5.8, J_{BX} 5.2, CH), 3.18 (1 H, dd, J_{AX} 5.8, J_{AB} 13.8, CHC*H*HPh) and 3.15 (1 H, J_{BX} 5.2, J_{AB} 13.8, CHCH*H*Ph); δ_C(125.7 MHz; [$^2H_4$]MeOH) 181.5 (CO), 140.0 (Ph C), 131.1 (Ph CH), 128.8 (Ph CH), 126.8 (Ph CH), 78.0 (CH) and 40.7 (CH*C*H_2Ph); $\delta_P\{^1H\}$ (101.26 MHz; water) 41.8; *m*/*z* (negative-ion electrospray) 261 [(M + 2H)$^-$, 16%], 243 [(M − O)$^-$, 11] and 227 [(M − S)$^-$, 100].

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