Phosphopeptide Prodrug Bearing an *S*-Acyl-2-thioethyl Enzyme-Labile Phosphate Protection

Christophe Mathé, Christian Périgaud,* Gilles Gosselin, and Jean-Louis Imbach

Laboratoire de Chimie Bioorganique, U.M.R. C.N.R.S. 5625, Case courrier 008, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

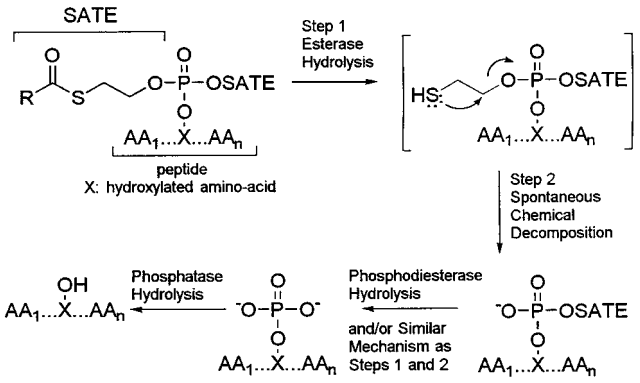
Received March 9, 1998 (Revised Manuscript Received September 10, 1998)

Introduction

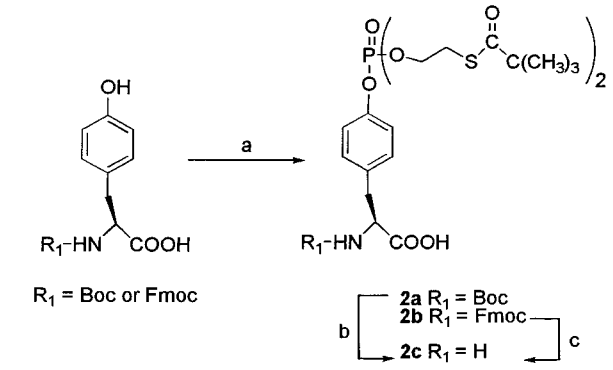
The synthesis of phosphopeptides and their analogues is a subject of considerable interest due to the importance of protein phosphorylation in eukaryotic cells. Indeed, phosphorylation and dephosphorylation of defined Ser, Thr, and/or Tyr residues in various proteins are involved in the regulation of different cellular processes and in signal transduction pathways.[1,2] Due to their significant role, phosphopeptides have received much attention as useful biological and biochemical tools in order to elucidate many cellular processes including signal transduction. Although the use of phosphorylated peptides and their analogues may lead to the development of biologically active agents as useful therapeutic drugs,[3,4] the clinical development of such compounds is hampered by their low permeability through biological barriers and by a lack of stability against enzymatic degradation.[5,6] One possible approach to improve the physicochemical properties of phosphopeptides is to use prodrug strategies to circumvent pharmaceutical and/or pharmacokinetic problems.[7,8] Recently, we described a promising approach in antiviral chemotherapy by using mononucleotide prodrugs, namely pronucleotides.[9,10] Thus, we have shown that mononucleoside phosphotriesters bearing *S*-acyl-2-thioethyl (SATE) groups were able to deliver selectively the corresponding nucleoside 5′-monophosphate in infected cells.

Applied to a phosphopeptide, the SATE prodrug strategy may enhance its membrane permeation characteristics and increase its stability against enzymatic degradation. After crossing the membrane barrier, the phosphopeptide prodrug should undergo an esterase-mediated activation process to generate an unstable thioethyl phosphotriester which decomposes spontaneously and selectively[11] (C−O bond cleavage) into the corresponding phosphodiester following an intramolecular nucleophilic substitution mechanism (Scheme 1). In a second step, the resulting phosphodiester could be converted, following a similar mechanism, into the phosphopeptide. Finally, once inside the cell the phosphopeptide can exhibit its biological effect and/or may act as a reservoir of the peptide after dephosphorylation by phosphatase activities. To illustrate the feasibility of the SATE prodrug strategy for phosphopeptides, we choose as a first model a pentapeptide, namely Leu-Enkephalinamide,[12,13] and we report here the synthesis and a preliminary enzymatic stability study of its derivative (**1**) (Scheme 3), incorporating a phospho-L-tyrosinyl residue protected by *S*-pivaloyl-2-thioethyl (*tert*-butylSATE) groups.

Scheme 1



Scheme 2



Reagents: (a) 1. TBDMSCl, DIEA, THF.2. bis(*S*-pivaloyl-2-thioethyl *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, THF. 3. *t*BuOOH. (b) 50% TFA in CH_2Cl_2. (c) 2% DBU in CH_2Cl_2.

Results and Discussion

Synthesis. Currently, two principal strategies are commonly used for the chemical synthesis of phospho-

* To whom correspondence should be addressed. Tel.: + (33) 4 67 14 47 76. Fax: + (33) 4 67 04 20 29. e-mail: perigaud@univ-montp2.fr.

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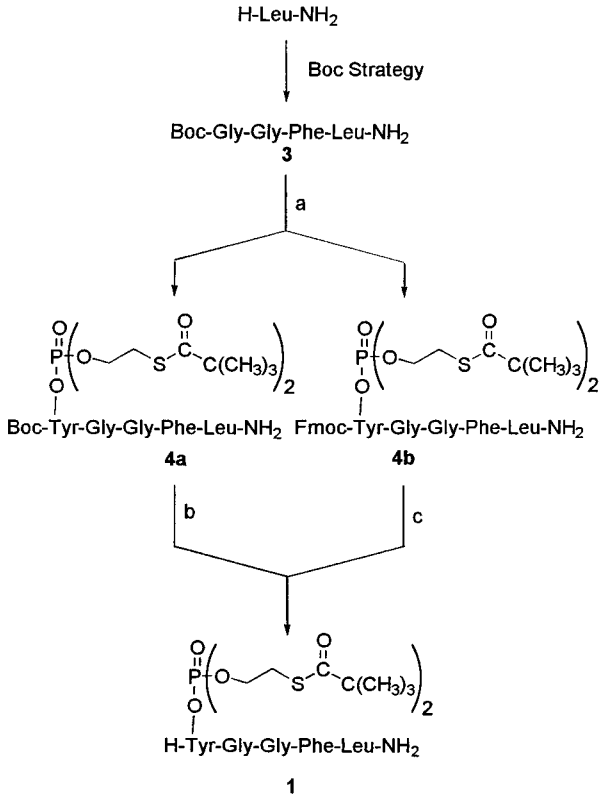
10.1021/jo980437d CCC: $15.00
Published on Web 10/27/1998

Scheme 3



Reagents: (a) 1. 50 % TFA in CH_2Cl_2 .2. **2a** or **2b**, BOP, DIEA, CH_2Cl_2. (b) 50 % TFA in CH_2Cl_2. (c) 2 % DBU in CH_2Cl_2.

rylated peptides: (a) the building-block strategy[14−20] where a protected phosphoamino acid is used during stepwise peptide assembly, and (b) the post-assembly method[21−26] where the phosphate group is added after peptide chain assembly. This latter approach is referred to a global phosphorylation. Phosphorylation step for the synthesis of the phosphoamino acid as well as the global phosphorylation of a peptide is based on P^{III} chemistry.[27] The chemical procedure can be carried out either in solid phase or in liquid phase following Boc or Fmoc strategy. For our purpose, we choose to investigate the feasibility to synthesize the target compound **1** by using the building-block strategy in liquid phase with Boc and Fmoc strategy. In this respect, the synthesis began with the preparation of the desired fully N-protected phosphotyrosine derivatives **2a**,**b** (Scheme 2). In this procedure, the *tert*-butyldimethylsilyl group was used for transient carboxyl protection,[28−30] and the protected Boc- or Fmoc-tyrosine intermediates, without isolation, were phosphitylated with bis(*S*-pivaloyl-2-thioethyl)-*N*,*N*-diisopropylphosphoramidite[31] and oxidized in situ by *tert*-butyl hydroperoxyde. The Boc- and Fmoc-phosphotyrosine derivatives **2a** and **2b** were obtained after purification by silica gel column chromatography[32] in 70 and 85% yield, respectively.

To determine the potential use in peptide synthesis of the new amino acid derivatives **2a**,**b** it was necessary to examine first the stability of the SATE phosphotriester structure under Boc and Fmoc cleavage conditions. Indeed, for other kinds of fully protected phosphotyrosine derivatives, premature cleavage with piperidine[25,33] or incomplete cleavage with TFA[33,34] and instability to strong acids, e.g., anhydrous HF,[34,35] have been reported. In our case, when **2a** was treated with 50% TFA in dichloromethane solution, complete removal of Boc group was observed within 5 min (monitored by HPLC), giving rise to the exclusive formation of the bis(*tert*-butylSATE) phosphotyrosinyl residue **2c**. In a similar manner, **2c** was obtained after treatment of **2b** with 2% DBU in dichloromethane solution.[25] Complete removal of Fmoc group was achieved within 10 min. In both cases, no decomposition of the SATE phosphotriester structure was observed, up to 1 h reaction time. It is noteworthy that these deprotection conditions are suitable for solid phase or solution phase peptide synthesis (based on a 10-min deprotection cycle).

To demonstrate the utility of both bis(*tert*-butylSATE) phosphotyrosinyl derivatives **2a**,**b** as building-blocks in a liquid-phase phosphotriester peptide synthesis, these compounds were used during a stepwise peptide assembly. The linear tetrapeptide Boc-Gly-Gly-Phe-Leu-NH_2 **3** was prepared using standard Boc strategy. After each coupling steps, the purity of the Boc peptide derivatives were checked by MS/FAB and ^1H NMR and then directly used in the next step without any further purification (Scheme 3). Coupling reaction of **3**, after its N-terminal deprotection, with either **2a** or **2b** was carried out using BOP[36] reagent with DIEA in dichloromethane to afford the Boc- and Fmoc-pentapeptide derivatives **4a**

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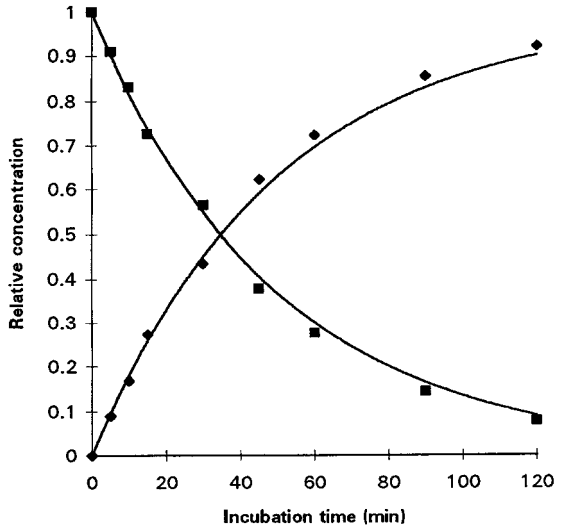

Figure 1. Stability of the (*tert*-butylSATE) phosphotriester derivative of Leu-enkephalinamide **1** in the presence of leucine aminopeptidase, at 37 °C, in phosphate buffer (pH 7.1). Curves shown the disappearance of **1** (■) and the appearance of the bis(*tert*-butylSATE) phosphotyrosinyl residue **2c** (◆) over a time period of 120 min.

and **4b** in 84 and 86% yield, respectively. Boc or Fmoc cleavage was accomplished with a solution of 50% TFA or 2% DBU in dichloromethane to give the desired phosphotriester pentapeptide **1**. The yield of the purified peptide **1** after Boc or Fmoc cleavage was 83 and 82%, respectively. The purity and identity of the phosphotyrosinyl peptide derivative **1** was unambiguously established by MS/FAB and ^{1}H and ^{31}P NMR.

Preliminary Enzymatic Stability. The opioid peptide Leu-Enkephalinamide is degraded in the central nervous system by neutral endopeptidase (EC 3.4.24.11)[37] and by leucine aminopeptidase (EC 3.4.11.2).[38] To check that the presence of the SATE phosphotriester structure on the N-terminal tyrosinyl residue would prevent (or at least slow) an enzymatic degradation, we choose to evaluate the stability of **1** against leucine aminopeptidase which is able to remove the N-terminal tyrosinyl residue of enkephaline derivatives.[39,40] The stability was determined at 37 °C in phosphate buffer (pH 7.1) with a ratio of enzyme/peptide 1/3 (w/w). The disappearance of **1** and the appearance of the bis(*tert*-butylSATE) phosphotyrosinyl residue **2c** were monitored by HPLC over a time period of 120 min (Figure 1). Under these conditions, **1** was degraded with an apparent half-life ($t_{1/2}$) of 34 min. Comparatively, the study was also carried out with Leu-enkephalinamide in identical conditions. In this case, the $t_{1/2}$ was lower than 1 min. These data show that the SATE phosphotriester structure confers to the parent peptide Leu-enkephalinamide a resistance to the enzymatic degradation by leucine aminopeptidase.

Conclusion

We have described the synthesis of a pentapeptide incorporating a bis(*S*-pivaloyl-2-thioethyl)-protected phospho-L-tyrosinyl residue. Our synthetic approach has allowed us to prepare in a suitable way the tyrosinyl phosphotriester derivative of Leu-Enkephalinamide in liquid phase using the building-block approach with Boc or Fmoc strategy. We have also demonstrated that this phosphopeptide prodrug shows increased leucine aminopeptidase stability as compared to the parent peptide. The extension of this methodology (e.g., solid phase synthesis, global phosphorylation, phosphoserine and phosphothreonine amino acids in place of phosphotyrosine) as well as its application to other biologically active phosphopeptides (e.g., SH_2 domain-directed peptides[41]) are currently under investigation.

Experimental Section

General Methods. Complete descriptions of apparatus and routine procedures used for the synthesis of the described compounds have recently been published.[42] All N-protected amino acid derivatives were purchased from Bachem. Leu-Enkephalinamide and leucine aminopeptidase (50 μg, lyophilized) were purchased from Sigma.

***N*-α-(*tert*-Butoxycarbonyl)-*O*-[bis(*S*-pivaloyl-2-thioethyl)]-L-phosphotyrosine (2a).** To a solution of Boc-Tyr-OH (0.6 g, 2.13 mmol) in dry THF (6 mL) was added *N*,*N*-diisopropyl-*N*-ethylamine (DIEA, 0.36 mL, 2.13 mmol) followed by *tert*-butyldimethylsilyl chloride (TBDMSCl, 0.38 g, 2.55 mmol). The solution was stirred at room temperature for 1 h. To this solution was added 1*H*-tetrazole (0.44 g, 6.4 mmol) and bis(*S*-pivaloyl-2-thioethyl)-*N*,*N*-diisopropylphosphoramidite (1.14 g, 2.55 mmol) in THF (3 mL). The resulting mixture was stirred at room temperature for 2 h, and then *tert*-butylhydroperoxide (*t*-BuOOH, 0.9 mL, 3 M solution in toluene) in CH_2Cl_2 (5 mL) was added. After being stirred for 30 min, solvents were removed, and the residue was dissolved in EtOAc (20 mL). The organic solution was washed with an aqueous solution of 5% $KHSO_4$ (6 mL) and water (3 × 30 mL) and dried over Na_2SO_4. The residue after evaporation was purified by silica gel column chromatography with a stepwise gradient of MeOH (0−12%) in CH_2Cl_2 to afford the compound **2a** as an oil (0.98 g, 70%): ^{1}H NMR (DMSO-d_6) δ 7.20 (d, 2H, J = 8.6 Hz), 7.07 (d, 2H, J = 8.4 Hz), 6.3 (bd s, 1H), 4.15 (q, 4H, J = 13.7 and 6.4 Hz), 3.9 (m, 1H), 3.14 (t, 4H, J = 6.3 Hz), 3.1−2.9 (m, 2H), 1.33 (s, 9H), 1.18 (s, 18H); ^{31}P NMR (DMSO-d_6) δ −5.78 (s); ^{13}C NMR (DMSO-d_6) δ 205.9, 175.1, 155.9, 149.2, 136.5, 131.4, 120.2, 78.6, 67.1, 56.4, 49.4, 46.8, 36.9, 28.9, 27.7; FAB MS (<0, G−T) *m*/*z* 648 $(M - H)^-$; $[\alpha]^{20}_D = +7$ (*c* 1.0, DMSO); HPLC t_R 24.2 min. Anal. Calcd for $C_{28}H_{44}NO_{10}PS_2$: C, 51.75; H, 6.82; N, 2.15. Found: C, 51.90; H, 6.93; N, 2.09.

***N*-α-(9-Fluorenylmethyloxycarbonyl)-*O*-[bis(*S*-pivaloyl-2-thioethyl)]-L-phosphotyrosine (2b).** To a solution of Fmoc-Tyr-OH (0.5 g, 1.24 mmol) in dry THF was added DIEA (0.21 mL, 1.24 mmol) followed by TBDMSCl (0.22 g, 1.49 mmol). The solution was stirred at room temperature for 3 h 30 min. To this solution was added 1*H*-tetrazole (0.26 g, 3.72 mmol) and bis(*S*-pivaloyl-2-thioethyl)-*N*,*N*-diisopropylphosphoramidite (0.68 g, 1.49 mmol) in THF (1.25 mL). The resulting mixture was stirred at room temperature for 3 h, and then *t*-BuOOH (0.54 mL, 3 M solution in toluene) in CH_2Cl_2 (3 mL) was added. After 20 min, the solvents were removed, and the residue was dissolved in EtOAc (15 mL). The organic solution was washed with an aqueous solution of 5% $KHSO_4$ (5 mL) and water (3 × 20 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography with a stepwise gradient of MeOH (0−10%) in CH_2Cl_2 to afford the compound **2b** as a white foam (0.82

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g, 85%): ^{1}H NMR (DMSO-d_6) δ 7.8−7.0 (m, 13H), 4.1−3.9 (m, 8H), 3.0 (m, 5H), 2.8 (m, 1H), 1.14 (s, 18H); ^{31}P NMR (DMSO-d_6) δ −5.95 (s); ^{13}C NMR (DMSO-d_6) δ 205.9, 174.4, 156.7, 149.4, 144.6, 141.5, 136.3, 131.1, 128.4, 127.9, 126.1, 120.9, 120.3, 67.1, 66.4, 56.6, 47.5, 46.8, 36.7, 29, 27.7; FAB MS (>0, G−T) *m/z* 772 $(M + H)^+$; FAB MS (<0, G−T) *m/z* 770 $(M - H)^-$; $[\alpha]^{20}_D$ = −3 (*c* 1.0, DMSO); HPLC t_R 28.3 min. Anal. Calcd for $C_{38}H_{46}NO_{10}PS_2$: C, 59.12; H, 6.01; N, 1.81. Found: C, 59.12; H, 5.99; N, 1.92.

***O*-[bis(*S*-pivaloyl-2-thioethyl)]-L-phosphotyrosine (2c).** From **2a**. Compound **2a** (100 mg, 0.15 mmol) was treated with a solution of trifluoroacetic acid (TFA) in CH_2Cl_2 (5 mL, 50:50, v/v) and stirred for 10 min. The solution was evaporated under reduced pressure and coevaporated several times with hexane. The residue was purified by silica gel column chromatography using a stepwise gradient of MeOH (0−40%) to afford **2c** as a white solid (62 mg, 87%): ^{1}H NMR (DMSO-d_6) δ 7.27 (d, 2H, J = 8.5 Hz), 7.10 (d, 2H, J = 8.2 Hz), 4.13 (q, 4H, J = 13.4 and 6.8 Hz), 3.4 (m, 1H, partially obscured by water), 3.1 (m, 5H), 2.8 (m, 1H), 1.14 (s, 18H); ^{31}P NMR (DMSO-d_6) δ −5.96 (s); ^{13}C NMR (DMSO-d_6) δ 205.9, 170.1, 149.6, 135.6, 131.7, 120.5, 67.1, 56.3, 46.9, 37.1, 28.9, 27.7; FAB MS (>0, G−T) *m/z* 642 $(M + G + H)^+$, 550 $(M + H)^+$, 145 $((CH_3)_3CC(O)S(CH_2)_2)^+$; FAB MS (<0, G−T) *m/z* 1097 $(2M - H)^-$, 640 $(M + G - H)^-$, 548 $(M - H)^-$; $[\alpha]^{20}_D$ = −7 (*c* 1.0, DMSO); HPLC t_R 22.1 min. Anal. Calcd for $C_{23}H_{36}NPO_8S_2$: C, 50.25; H, 6.60; N, 2.55. Found: C, 50.31; H, 6.85; N, 2.66.

From **2b**. Compound **2b** (70 mg, 0.09 mmol) was treated with a solution of 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (1.5-*S*) (DBU) in CH_2Cl_2 (3.5 mL) and stirred for 10 min. The solution was evaporated under reduced pressure. The residue was purified by column chromatography using a stepwise gradient of MeOH (0−40%) in CH_2Cl_2 to give **2c** as a white solid (40 mg, 80%). The physicochemical properties were identical to those obtained from **2a** in Boc cleavage conditions.

Peptide Derivative 4a. The tetrapeptide Boc-Gly-Gly-Phe-Leu-NH_2 **3** (150 mg, 0.30 mmol) was added to a solution of TFA/CH_2Cl_2 (2 mL, 50:50, v/v) and stirred for 30 min. The solution was evaporated under reduced pressure and coevaporated several times with hexane. The residue was dissolved in CH_2Cl_2 (4 mL) and then DIEA (0.26 mL, 1.52 mmol), BOP reagent (135 mg, 0.37 mmol), and compound **2a** (200 mg, 0.31 mmol) were successively added. The solution was stirred at room temperature for 2 h, and the solvent was evaporated under reduced pressure. The oily residue was dissolved in EtOAc (50 mL), washed with an aqueous solution of 5% $KHSO_4$ (4 × 50 mL), dried over Na_2SO_4, and concentrated to dryness in vacuo. Purification by silica gel column chromatography using a stepwise gradient of MeOH (0−5%) in CH_2Cl_2 afforded compound **4a** (264 mg, 84%) as an oil: ^{1}H NMR (DMSO-d_6) δ 8.2 (m, 1H), 8.1 (m, 2H), 7.9 (m, 1H), 7.2−6.9 (m, 12H), 4.5 (m, 1H), 4.1 (m, 6H), 3.7−3.6 (m, 4H), 3.12 (t, 4H, J = 6.3 Hz), 3.0−2.7 (m, 4H), 1.55 (m, 1H), 1.45 (m, 2H), 1.26 (s, 9H), 1.16 (s, 18H), 0.84 (dd, 6H); ^{31}P NMR (DMSO-d_6) δ −6.0 (s); FAB MS (0<, G−T) *m/z* 1023 $(M + H)^+$, 145 $((CH_3)_3CC(O)S(CH_2)_2)^+$, 120 $(PhCH_2CH{=}NH_2)^+$; FAB MS (<0, G−T) *m/z* 1021 $(M - H)^-$.

Peptide Derivative 4b. The tetrapeptide Boc-Gly-Gly-Phe-Leu-NH_2 **3** (150 mg, 0.30 mmol) was added to a solution of TFA/CH_2Cl_2 (2 mL, 50:50, v/v) and stirred for 30 min. The solution was evaporated under reduced pressure and coevaporated several times with hexane. The residue was dissolved in CH_2Cl_2 (4 mL), and then DIEA (0.26 mL, 1.52 mmol), BOP reagent (135 mg, 0.37 mmol), and compound **2b** (254 mg, 0.33 mmol) were successively added. The solution was stirred at room temperature for 2 h and then evaporated under reduced pressure. The oily residue was dissolved in EtOAc (50 mL), washed with 5% $KHSO_4$ (4 × 50 mL), dried over Na_2SO_4, and concentrated to dryness in vacuo. Purification by silica gel column chromatography using a stepwise gradient of MeOH (0−5%) in CH_2Cl_2 afforded compound **4b** (300 mg, 86%) as an oil: ^{1}H NMR (DMSO-d_6) δ 8.3−6.9 (m, 24 H), 4.49 (m, 1H), 4.2−4.0 (m, 9H), 3.7−3.5 (m, 4H), 3.1−2.9 (m, 6H), 2.7 (m, 2H), 1.5 (m, 1H), 1.4 (m, 2H), 1.14 (s, 18H), 0.83 (dd, 6H); ^{31}P NMR (DMSO-d_6) δ −5.99 (s); FAB MS (0<, G−T) *m/z* 1145 $(M + H)^+$, 145 $((CH_3)_3CC(O)S(CH_2)_2)^+$, 120 $(PhCH_2CH{=}NH_2)^+$, 86 $((CH_3)_2CHCH_2CH{=}NH_2)^+$, 30 $(CH_2{=}NH_2)^+$; FAB MS (<0, G−T) *m/z* 1143 $(M - H)^-$.

***tert*-butylSATE Phosphotriester Derivative of Leu-Enkephalinamide (1). Boc Cleavage Conditions.** The Boc peptide phosphotriester **4a** (75 mg, 0.07 mmol) was treated with a solution of TFA/CH_2Cl_2 (0.8 mL, 50:50, v/v) and anisole (0.04 mL, 0.37 mmol). After 15 min, addition of Et_2O led to a formation of a white precipitate. The precipitate was collected by filtration, washed several times with Et_2O, and purified by silica gel column chromatography using a stepwise gradient of MeOH (0−15%) in CH_2Cl_2 to afford the trifluoroacetate salt of the target compound **1** as a white solid (65 mg, 83%): ^{1}H NMR (DMSO-d_6) δ 8.8 (m, 1H), 8.18 (m, 1H), 8.1−7.9 (m, 5H), 7.3−7.1 (m, 10H), 6.90 (s, 1H), 4.5 (m, 1H), 4.13 (m, 5H), 4.05 (m, 1H), 3.8−3.6 (m, 4H), 3.13 (t, 4H, J = 6.3 Hz), 3.1−2.7 (m, 4H), 1.55 (m, 1H), 1.44 (m, 2H), 1.17 (s, 18H), 0.84 (dd, 6H); ^{31}P NMR (DMSO-d_6) δ −6.03 (s); FAB MS (0<, G−T) *m/z* 923 $(M + H)^+$, 793 $(M - \text{Leu-}NH_2)^+$, 589 $(M - \text{Gly-Phe-Leu-}NH_2)^+$, 145 $((CH_3)_3CC(O)S(CH_2)_2)^+$, 120 $(PhCH_2CH{=}NH_2)^+$, 86 $((CH_3)_2CHCH_2CH{=}NH_2)^+$, 30 $(CH_2{=}NH_2)^+$; FAB MS (<0, G−T) *m/z* 1035 $(M + \text{TFA} - H)^-$, 921 $(M - H)^-$. HRMS: calcd for $C_{42}H_{64}N_6O_{11}PS_2$ 923.3812, found 923.3828.

Fmoc Cleavage Conditions. The Fmoc peptide phosphotriester **4b** (200 mg, 0.17 mmol) was treated with a solution of 2% DBU in CH_2Cl_2 (2.5 mL). After 15 min, addition of Et_2O led to a formation of white precipitate. The precipitate was collected by filtration, washed several times with Et_2O, and purified by chromatography using a stepwise gradient of MeOH (0−15%) in CH_2Cl_2 to afford the target compound **1** as a white solid (133 mg, 82%): ^{1}H NMR (DMSO-d_6) δ 8.2 (m, 1H), 8.1−8.0 (m, 2H), 7.3−7.0 (m, 10H), 6.98 (s, 2H), 4.47 (m, 1H), 4.13 (m, 5H), 3.8−3.6 (m, 4H), 3.4 (m, 1H, partially obscured by water), 3.12 (t, 4H, J = 6.2 Hz), 3.0−2.5 (m, 4H), 2.0 (m, 2H), 1.55 (m, 1H), 1.46 (m, 2H), 1.07 (s, 18H), 0.84 (dd, 6H); ^{31}P NMR (DMSO-d_6) δ −5.94 (s); FAB MS (0<, G−T) *m/z* 923 $(M + H)^+$, 793 $(M - \text{Leu-}NH_2)^+$, 589 $(M - \text{Gly-Phe-Leu-}NH_2)^+$, 145 $((CH_3)_3CC(O)S(CH_2)_2)^+$, 120 $(PhCH_2CH{=}NH_2)^+$, 86 $((CH_3)_2CHCH_2CH{=}NH_2)^+$; FAB MS (<0, G−T) *m/z* 921 $(M - H)^-$. HRMS: calcd for $C_{42}H_{64}N_6O_{11}PS_2$ 923.3812, found 923.3834.

Acknowledgment. These investigations were supported by grants from CNRS and "Agence Nationale de Recherches sur le SIDA" (ANRS, France). One of us (C.M.) is particularly grateful to ANRS for a postdoctoral fellowship.

Supporting Information Available: The detailed synthesis of **3**, a full listing of NMR data accompanied by subjective peak assignments, and procedures used for the stability study of SATE phosphotriester structures **2a**,**b** and the preliminary enzymatic stability of **1** (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO980437D