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N,N-Diisopropyl-bis[2-(trimethylsilyl)ethyl]phosphoramidite. An Attractive Phosphitylating Agent Compatible with the Fmoc/t-Butyl Strategy for the Synthesis of Phosphotyrosine Containing Peptides

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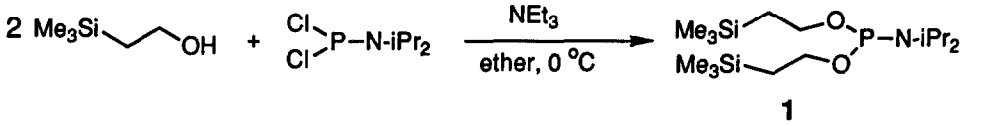
Abstract: A new phosphitylating agent, compatible with the Fmoc/t-butyl strategy for the production of phosphotyrosine containing peptides was synthesized. Our results demonstrated that high yields and high purities of phosphotyrosine peptides can be obtained using this phosphitylating agent.

Tyrosine-specific protein phosphorylation has been recognized as an important cell regulatory process.[1] It has been reported that tyrosine specific protein kinases are involved in the cellular response to the epidermal growth factor, insulin and platelet-derived growth factor.[2] Although protein tyrosine phosporylation has been proposed to play an important part in many biological processes, the modes of action are still somewhat unclear. Therefore synthetic phosphotyrosine containing peptides may serve as a valuable tool to study the influence of phosphorylation on structure and function at the molecular level.

In general, there are two chemical methodologies to synthesize phosphotyrosine containing peptides. (a) The building-block approach[3] (i.e. using protected phosphotyrosine for peptide synthesis) and (b) the post-assembly approach[3a,b,i,4] (i.e. the free tyrosine side chain phenol group in a presynthesized, resin-bound peptide is phosporylated by a suitable phosphitylating agent followed by oxidation to give a resin-bound fully protected phosphotyrosine peptide). The building block approach may be advantageous in cases targeting peptides containing oxidizable amino acids such as Met, Cys and Trp. The post-assembly approach, in contrast is usually very straightforward and more flexible but side reactions involving oxidation of Met, Cys, Trp are possible.[4a,b] An advantage, however, is that from a single synthesis, two peptides (phosphorylated and unphosphorylated) and possibly other analogs can be obtained.

In this account we like to report a new phosphitylating agent, compatible with the Fmoc/t-butyl solid phase synthesis strategy for production of phosphotyrosine containing peptides, based on 2-(trimethylsily)ethanol. The trimethylsilylethyl group has been used for nitrogen[5] and oxygen[6] protection. In addition to deblocking by fluoride ion, the trimethylsilyl ethyl group can also be removed by treatment with TFA.[5] Such an interesting property renders the trimethylsilyl ethyl moiety particularly attractive as a potential masking group for phosphite oxygen protection using Fmoc/t-butyl based solid phase peptide synthesis, because during the final cleavage and deprotection of the protected peptidyl resin samples, the trimethylsilyl ethyl group is simultaneously cleaved under normal conditions.

N,N-Diisopropyl-bis[2-(trimethylsilyl)ethyl]phosphoramidite **1** was prepared in one step from commercially available starting materials dichloro(diisopropylamino)phosphine and 2-(trimethylsilyl)ethanol following a literature procedure[7] to give the desired product as a clear liquid in 65% yield, after purification by column chromatography eluting with hexane/EtOAc/triethylamine 85:15:4 (eq. 1).[8]

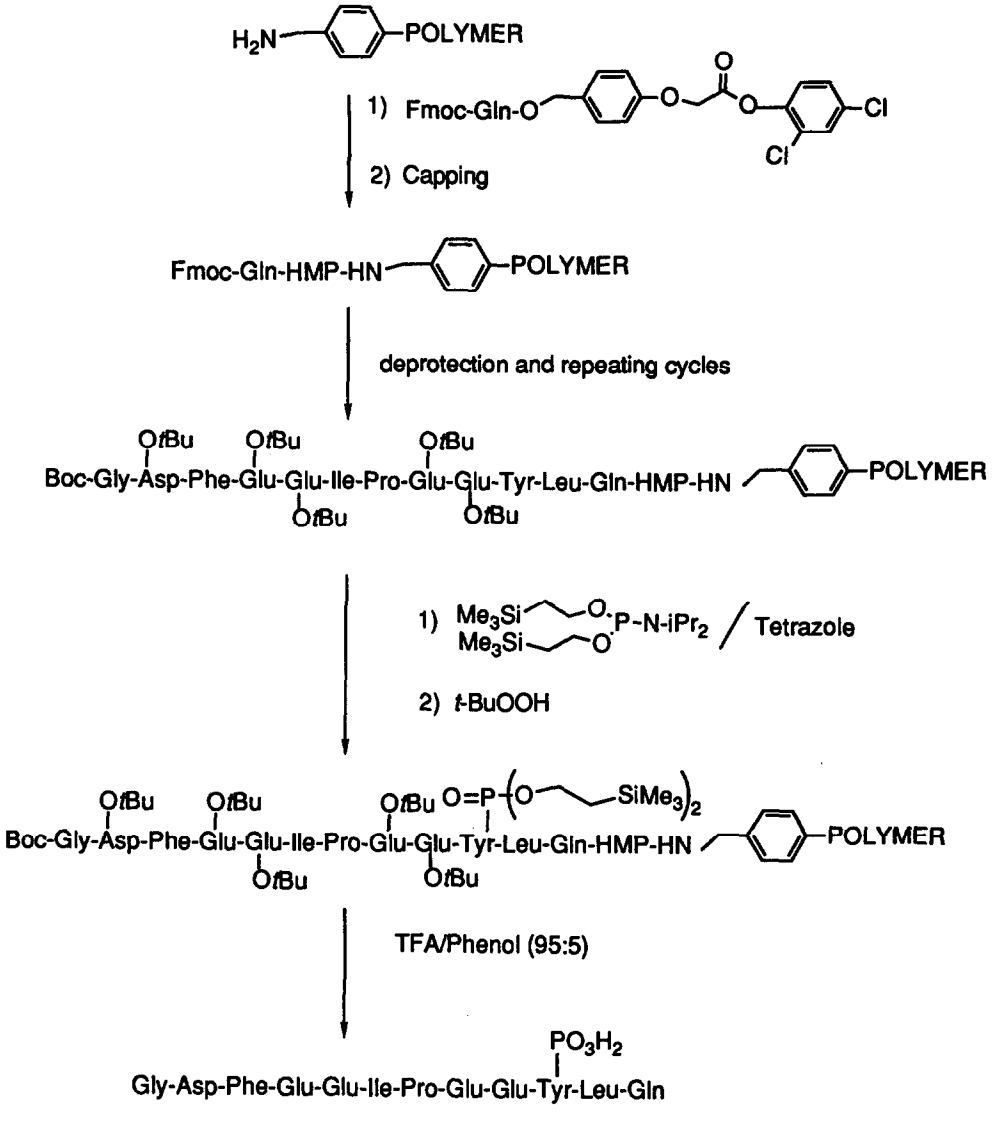


1

eq 1

In order to demonstrate the utility of the new phosphitylating agent **1**, two peptides YLRVA **2** (residues 1174-1178 of the human EGF receptor) and GDFEEIPEEYLQ **3** (Hirudin 54-65) were chosen for the study. Peptide **2** was prepared using Fmoc-Ala-Pepsyn KA^{TM} resin (2.2 g, 0.2 mmol; 0.09 mmol/g loading) on an automated continuous flow peptide synthesizer using a standard Fmoc/t-butyl protocol. Arginine was protected by the Mtr group and tyrosine was coupled as Boc-Tyr-OH. After synthesis was complete, the peptidyl-resin sample was washed and dried *in vacuo* overnight and 1g of the peptidyl-resin sample was phosphorylated with 10 equiv. of the phosphitylating agent **1** and 50 equiv. of tetrazole in DMA for 1 h followed by oxidation with 20 equiv. tert-BuOOH in DMA for 1.5 h. After cleavage with 10 ml TFA/phenol/DTT solution (95:2.5:2.5) for 6 h, the crude peptide **2** was isolated by ether precipitation and was further purified by RP-HPLC to yield 54.1mg of the homogeneous phosphotyrosine peptide as di-TFA salt in 55% yield. The identity of the phosphopeptide was established by FAB/MS, $(M + H)^+ = 701$. Peptide **3** was synthesized manually (Scheme 1) using Fmoc-Gln-HMPA-aminomethylpolystyrene (434mg, 0.2 mmol; 0.46 mmol/g loading) which was prepared using the preformed 2,4-dichlorophenyl ester of the Fmoc-Gln-phenoxyacetic acid synthesized as described by Bernatowicz et al.[9] It was coupled onto aminomethylpolystyrene as follows: 0.6 equiv. of the ester and 1 equiv. of aminomethylpolystryene (3.38 mmol-NH_2/g) in the presence of 5 equiv. of pyridine in DMF for 2-3 h. The unreacted amino groups were then capped by reaction with acetic anhydride and pyridine in DMF and the resulting resin was subject to Fmoc-UV analysis[9] to give 0.46 mmol/g substitution level of Fmoc function. Peptide chain elongation was carried out using 4 equiv. of preformed Fmoc-AA-Pfp esters in the presence of an equimolar amount of HOBt in DMF, except Fmoc-Tyr-OH and Boc-Gly-OH which were coupled using BOP[10] reagent. Deprotection of the Fmoc group was performed using 20% piperidine/DMF. After incorporation of the last amino acid, the peptidyl-resin was treated with 20% piperidine/DMF for 15 min, washed (DMF, HOAc, MeOH, Ether) and dried *in vacuo* overnight. A 100 mg of the peptidyl-resin sample was phosphorylated according to the procedure described for peptide **2**. Cleavage of the peptidyl-resin sample with 10 ml of TFA/Phenol solution (95:5, 2h) yielded a crude peptide which was purified by RP-HPLC (Fig. 1) to give 45.2 mg of the homogeneous phosphotyrosine peptide in 54.3% yield. FAB/MS, $(M+ H)^+ = 1548.6$, confirmed the identity of the product.

In conclusion, we have demonstrated the utility of the new phosphitylating agent **1** for production of phosphotyrosine containing peptides and also expected that the phosphitylating agent **1** should be suitable for making phosphoserine and phosphothreonine containing peptides using the post assembly approach in general.



Scheme 1. Synthesis and phosphorylation of Hirudin 54-65 peptide using phosphitylating agent 1

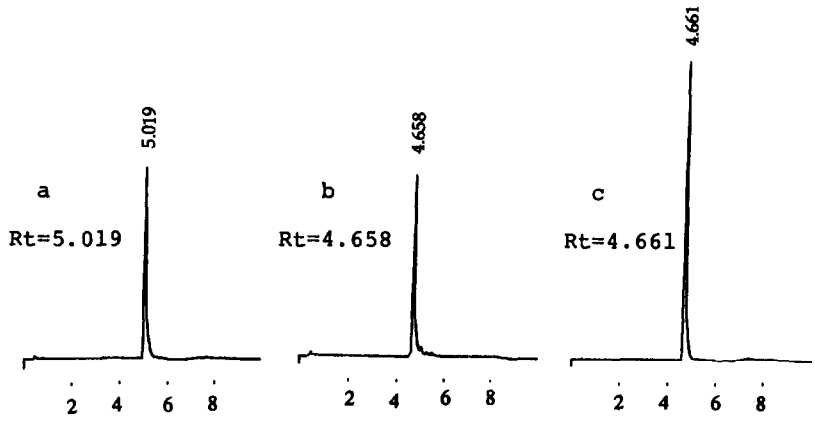


Fig. 1 HPLC chromatograms of (Hirudin 54-65) 3 GDFEEIPEEYLQ. HPLC conditions: YMC ODS-AQ column, 4 mm x 50 mm, 3 um particle, f = 2 ml/min, 220 nm, eluent A, 0.1% (w/v) TFA in H_2O; B, 0.1% (w/v) TFA in CH_3CN containing 5% A, gradient 6-64% B in 6 min. (a) crude unphosphorylated peptide. (b) crude phosphorylated peptide. (c) purified phosphorylated peptide.

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8. ^{1}H NMR ($CDCl_3$) δ 0.0 (s, 18 H), 1.02 (m, 4 H), 1.08 (d, 12 H), 3.52-3.83(m, 6 H); ^{31}P NMR ($CDCl_3$) 143.55 ppm; ^{13}C NMR ($CDCl_3$) -1.49, 19.97, 20.05, 24.41, 24.51, 42.47, 42.63, 60.43, 60.67 ppm; FAB/MS $(M + H)^+ = 366$.
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