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SOLID PHASE SYNTHESIS OF PEPTIDES CONTAINING A PHOSPHOSERINE - SULFUR MUSTARD ADDUCT

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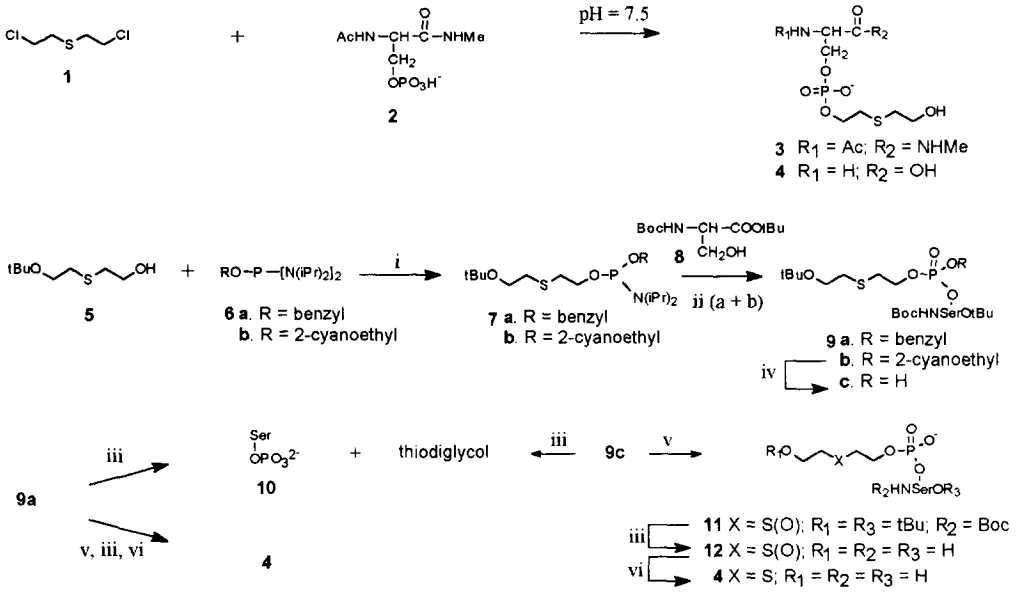
Abstract. Solid phase synthesis of peptides containing a serine thiodiglycol phosphate residue has been achieved by following a global on-resin phosphorylation strategy with a functionalized benzyl phosphoramidite. To preclude cleavage of the acid labile thiodiglycol phosphate ester during deprotection, the thioether functionality was temporarily protected as a sulfoxide.

Sulfur mustard (**1**; Scheme 1) is a powerful vesicant, cytotoxic agent and primary carcinogen used as a chemical warfare agent since World War I. Due to the strong electrophilic character of its episulfonium ion it binds extensively to nucleic acids and proteins. We are engaged in the development of methods for retrospective detection of exposure to **1**[1-4]. In addition, we are interested in the pathologic mechanisms underlying sulfur mustard induced skin vesication and cell death. Since mustards exhibit high reactivity towards phosphate monoesters[2,5], we reasoned that sulfur mustard might have a profound effect on protein phosphorylation/dephosphorylation, which is one of the major regulation mechanisms of cellular processes as glycogen metabolism[6], ion channel permeability[7] and signal transduction[8]. For instance, alkylation of phosphoamino acid residues might prevent dephosphorylation by phosphatases which may have *de facto* the same effect as phosphatase inhibitors such as thiodiglycol[9] and the natural vesicant cantharidin[10]. Moreover, phosphate alkylation may affect cell membrane integrity.

Preliminary experiments in our laboratory demonstrated that reaction of **1** with phosphoserine derivative **2** in aqueous solution at pH 7.5, resulted in the rapid formation of phosphodiester **3** (Scheme 1). We are interested in peptides containing serine thiodiglycol phosphate (**4**) since these may be used to assess the toxicological significance of (**4**), *e.g.*, the inhibition of protein dephosphorylation. In addition, antibodies raised against peptide haptens containing **4** might be useful tools to locate cellular damage after exposure to **1**. These antibodies may also be useful for verification of exposure to **1**, since phosphoserine is a major component of keratin, which is one of the most abundant proteins in skin[11]. In this paper we present the solid phase synthesis of peptides containing **4** by following a global on-resin phosphorylation strategy.

Much effort has been devoted towards the organic synthesis of well-defined phosphopeptides[12]. A general procedure for their synthesis comprises the on-resin phosphitylation of preformed peptides with dibenzyl-(*N,N*-diisopropyl)phosphoramidite, followed by oxidation of the intermediate phosphite (global strategy)[13]. We anticipated that this approach might be valid for the solid phase synthesis of peptides containing **4**, using the functionalized phosphoramidite **7a** (Scheme 1) as the phosphitylating reagent[14]. The latter could be obtained by 1*H*-tetrazole mediated reaction of thiodiglycol derivative **5**[15] with easily accessible benzyl-*bis*(*N,N*-diisopropyl)phosphordiamidite **6a**[16]. Purification of the crude product by flash column chromatography on silica gel (eluent: n-hexane/Et_3N, 99/1, v/v, to n-hexane/Et_3N, 97/3, v/v) gave homogeneous **7a**[17] in moderate yield (40%), which could be stored (-20 °C) for months without significant decomposition.

Scheme 1



Key: (i) 1*H*-tetrazole (1.1 eq), CH_2Cl_2/CH_3CN (4/1, v/v), 3 h (40% for **7a**; 66% for **7b**). (iia) 1*H*-tetrazole (3 eq), CH_2Cl_2/CH_3CN (4/1, v/v), 0.5 h; (iib) 80% tBuOOH (5 eq), CH_2Cl_2/CH_3CN (4/1, v/v), 0.75 h, 0 °C (45%). (iii) TFA/H_2O (95/5, v/v), 2 h. (iv) Et_3N/pyridine/H_2O (10/85/5, v/v/v), 16 h (79%). (v) H_2O_2 (10 eq), AcOH/H_2O/CH_3CN (0.2/1/4, v/v/v). (vi) N-methylmercapto-acetamide (10 eq), AcOH/H_2O (5/95, v/v), 72 h, 45 °C.

In order to establish the usefulness of reagent **7a**, the solution phase synthesis of **4** was attempted. Thus, N-α-Boc-L-serine *tert*-butyl ester (**8**) was reacted with **7a** (1.0 eq) under the agency of 1*H*-tetrazole, followed by oxidation of the intermediate phosphite triester with tBuOOH. After work-up and silica gel column chromatography (eluent: CH_2Cl_2 to CH_2Cl_2/acetone, 9/1, v/v) serine derivative **9a** could be isolated as a mixture

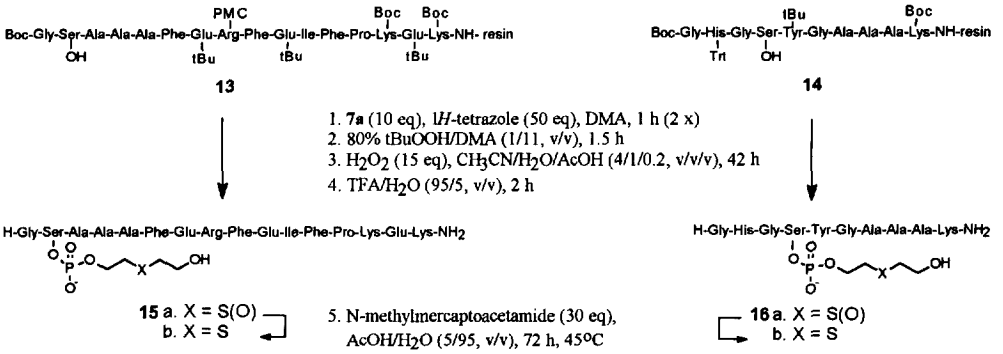
of diastereomers in 45% yield. Its structure was firmly corroborated by NMR spectroscopy[17]. No evidence for the formation of a sulfoxide-containing product was obtained.

To our dismay, deprotection of **9a** with TFA/H_2O (95/5, v/v) did not give the target molecule **4**, but resulted in a mixture of serine phosphate (**10**) and thiodiglycol, as was evidenced by NMR spectroscopy. Application of alternative deprotection conditions (neat TFA; TFA/thioanisole) resulted also in the formation of **10**. Presumably, the driving force of this acid catalyzed hydrolysis is the formation of a stable episulfonium ion. In order to check whether the rate of formation of this episulfonium ion could be reduced, the acidic deprotection step was performed in a phosphodiester instead of a phosphotriester stage. To this purpose phosphodiester **9c** was prepared via the corresponding cyanoethyl-triester **9b**, which was readily obtained by reaction of **8** with phosphoramidite 7**b**. However, deblocking (TFA/H_2O; 95/5, v/v) of **9c** also led to thiodiglycol release.

We argued that the stability of this acid-labile phosphate ester would be greatly increased when the thioether function was incorporated as a sulfoxide, thereby precluding the formation of a stable episulfonium ion. Unfortunately, attempts to synthesize the sulfoxide analog of phosphoramidite **7a**, via reaction of **6a** with the sulfoxide analog of **5**, were abortive. Probably, the polarized sulfoxide function reacts rapidly with **6a** by nucleophilic attack on phosphorus, giving a product which decomposes during isolation. We then turned our attention towards the introduction of the sulfoxide function in a later synthetic stage. In a first attempt[18], phosphodiester **9c** (10 mg) was treated with 30% H_2O_2/AcOH (1/1, v/v; 40 µl) in CH_3CN/H_2O (4/1, v/v; 500 µl) for 4 h, after which ^1H NMR spectroscopy showed a clear downfield shift of the methylene signals adjacent to sulfur, indicating conversion into sulfoxide **11**. When **11** was treated with TFA/H_2O (95/5, v/v), ^{31}P NMR showed quantitative conversion into a compound showing two equal peaks (diastereomeric mixture)[17], which indicates that the 2,2'-sulfinyl*bis*-ethanol-phosphate moiety was unimpaired. Subsequent reduction of **12** was performed with N-methylmercaptoacetamide (MMA) in 5% aqueous acetic acid, as has been described for methionine sulfoxide residues in peptides and proteins[19]. Although reduction proceeded rather sluggishly, ^{31}P-NMR spectroscopy showed quantitative conversion into a single compound (**4**)[17]. Apparently, the acid-labile thiodiglycol phosphate ester is stable enough to survive these slightly acidic conditions. An aliquot of crude **4** was purified by gel filtration (Sephacryl S-100), followed by anion-exchange chromatography (DEAE A-25). Purified **4** gave satisfactory mass spectrometric and NMR data[17] and proved stable in aqueous solution. Following the same strategy, compound **4** could also be obtained from benzyl phosphotriester **9a**. Since this route precludes the possibility of base-catalyzed β-elimination of the serine moiety, **7a** was selected for the phosphorylation of immobilized peptides.

The viability of the developed phosphorylation strategy was demonstrated in the global phosphorylation of immobilized peptides **13** and **14** (see Scheme 2), which were assembled on solid phase (Tentagel S-AM resin; 0.27 mmol/g loading) by application of standard Fmoc/*tert*-Bu chemistry, the serine residue being incorporated without side-chain protection and the N-terminal glycine residue being incorporated with Boc protection. The

Scheme 2



intended peptide **15b** represents the phosphoserine adduct **4** capped with a glycine residue at the N-terminus and coupled via a spacer (Ala-Ala-Ala) to a T-cell epitope[20] in order to enhance immunogenic response, while peptide **16b** mimics a small part of the human keratin 1 chain[21] alkylated at phosphoserine-576 and coupled to a spacer (Ala-Ala-Ala-Lys-NH_2) for attachment to a carrier protein. Prior to phosphorylation the resins were extensively dried on P_2O_5. Phosphorylation of **13** and **14** was carried out as follows. The resin (50 mg, containing 13 μmol peptide) was swollen in anhydrous DMA (100 μL) for 1 h, after which a solution of **7a** (10 eq) and 1*H*-tetrazole (50 eq) in DMA (100 μL) was added. After 1 h, the phosphitylation step was repeated. The resin was washed with DMA (5x 1 ml) and oxidation of the phosphite was effected by addition of a solution of 80% tBuOOH (0.175 ml) in DMA (2 ml). After 1.5 h, the resin was washed with DMA (5x 1 ml) and oxidation of the thioether function was accomplished by addition of a solution of 30% H_2O_2 (20 μL; 15 eq) in CH_3CN/H_2O/AcOH (4/1/0.2, v/v/v; 0.5 ml). To assure complete oxidation the mixture was left for 42 h, after which the resin was washed with DMA (5x 1 ml) and diethyl ether (5x 1 ml). After cleavage and deprotection by treatment with TFA/H_2O (95/5, v/v), the crude peptides were purified by FPLC (PepRPC 5/5), affording **15a** and **16a** (see Figure 1b-c for crude and purified **16a** and Figure 1a for corresponding unphosphorylated sequence), which gave satisfactory mass spectrometric data[17]. Reduction was accomplished as described for compound **12**, after which purification yielded homogeneous **15b** and **16b**, as evidenced by FPLC (see Figure 1d for **16b**) and mass spectrometric analysis (see Figure 2 for **15b**).

In conclusion, the synthesis of peptides containing the phosphoserine - sulfur mustard adduct **4** has been achieved by following a global on-resin phosphorylation strategy using phosphoramidite **7a**. It was established that cleavage of the acid-labile thiodiglycol phosphate linkage could be precluded by temporary protection of the thioether functionality as a sulfoxide. At present, immunochemical experiments with **15b** and **16b** are in progress.

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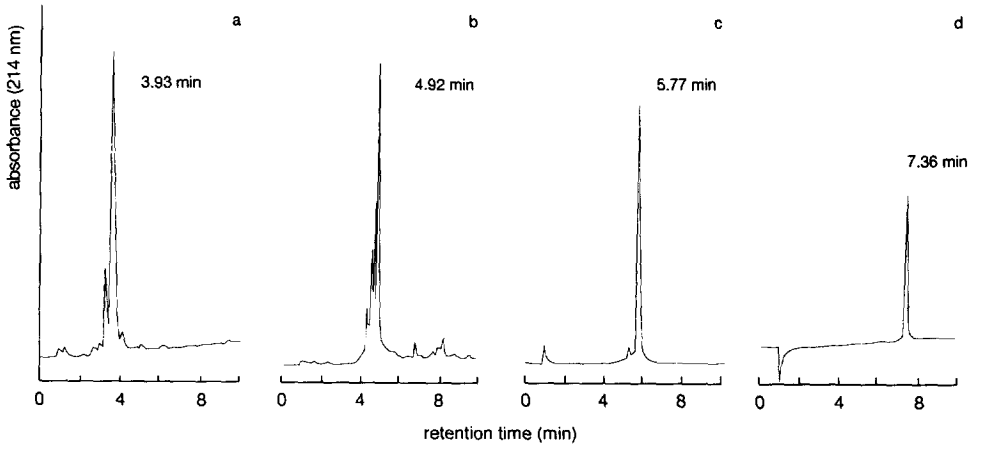

Figure 1. FPLC analysis of crude and purified peptides. a) crude, deprotected **14** after cleavage from resin; b) crude **16a**; c) purified **16a**; d) purified **16b**. Conditions FPLC: PepRPC 5/5 column. Eluents: (A) 0.1% TFA/H_2O; (B) 0.1% TFA in CH_3CN/H_2O (70/30, v/v). Flow rate 1.0 ml/min. Detection: 214 nm. Gradient for chromatograms a + b: 0 - 60% B in 20 min. Gradient for chromatograms c + d: 0 - 30% B in 20 min.

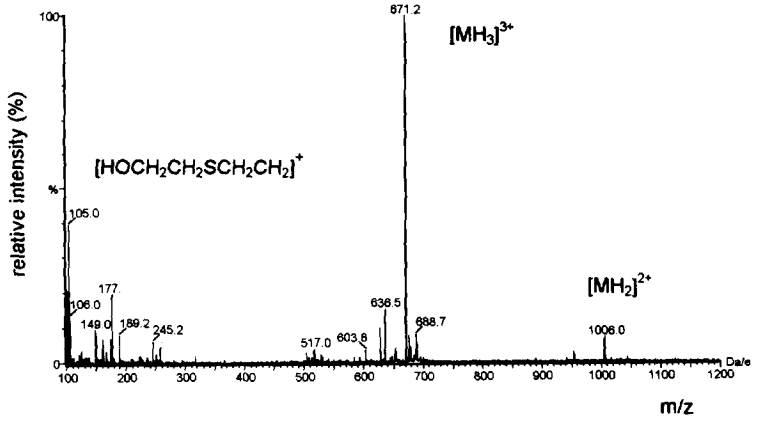

Figure 2. Electrospray (positive ion mode) mass spectrum of purified **15b**.

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14. The compatibility of the thioether group with tBuOOH had been ascertained by incubation of **5** with tBuOOH (10 eq) in CH_3CN. After 16 h, no oxidation of sulfur had occurred, as was demonstrated by NMR spectroscopy.
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17. All new compounds gave satisfactory analytical data:
4: ^{31}P NMR (D_2O): δ 0.75. ^1H NMR (D_2O): δ 2.70 - 2.90 (2x t, 4H, 2x CH_2S), 3.70 (t, 2H, CH_2OH), 3.80 (m, 1H, Hα-serine), 4.05 (m, 2H, $POC\underline{H}_2CH_2$), 4.00 - 4.10 (m, 2H, CH_2-serine). ^{13}C NMR (D_2O): δ 32.4 (d, $POCH_2\underline{C}H_2$; J_{PC} = 8.1 Hz), 34.5 (CH_2S), 56.2 (Cα-serine; J_{PC} = 5.8 Hz), 61.2 (CH_2OH), 66.0 ($PO\underline{C}H_2CH_2$; J_{PC} = 5.9 Hz), 66.3 (CH_2-serine; broad). Electrospray MS (positive ion mode): *m/z* 312.2 $[M + Na]^+$, 290.2 $[MH]^+$, 105.1 $[HOCH_2CH_2SCH_2CH_2]^+$. Electrospray MS (negative ion mode): 288.2 $[M-H]^-$, 201.1 $[HOCH_2CH_2SCH_2CH_2OPO_3H]^-$. **7a**: ^{31}P NMR ($CDCl_3$): δ 147.9. ^1H NMR ($CDCl_3$): δ 1.18 + 1.20 (2x d, 12H, CH_3-iPr); 1.19 (s, 9H, CH_3-tBu); 2.67 + 2.78 (2x t, 4H, 2x CH_2S), 3.50 (t, 2H, CH_2O-tBu), 3.60-3.70 (m, 2H, CH-iPr), 3.75-3.90 (m, 2H, $POC\underline{H}_2CH_2$), 4.60-4.80 (m, 2H, CH_2-benzyl), 7.20-7.40 (m, 5H, H-aromatic). ^{13}C NMR ($CDCl_3$): δ 24.7 (CH_3-iPr, J_{PC} = 7.2 Hz); 27.7 (CH_3-tBu), 33.4 (CH_2S); 33.7 ($POCH_2\underline{C}H_2S$, J_{PC} = 6.9 Hz); 43.2 (CH-iPr, J_{PC}=12.5 Hz); 62.1 (CH_2O-tBu); 63.4 ($PO\underline{C}H_2CH_2$, J_{PC}=17.5 Hz); 65.5 (CH_2-benzyl, J_{PC}=18.1 Hz); 73.3 ($\underline{C}(CH_3)_3$); 127.0-128.0 (C-aromatic); 139.6 (C_q). **7b**: ^{31}P NMR ($CDCl_3$): 148.7. **9a**: ^{31}P NMR ($CDCl_3$): δ -0.70. ^1H NMR ($CDCl_3$): δ 1.10 - 1.50 (3x s, 27H, CH_3-tBu); 2.60 - 2.90 (2x t, 4H, 2x CH_2S); 3.50 (t, 2H, CH_2O-tBu); 4.10 (m, 2H, $POC\underline{H}_2CH_2$); 4.25 (m, 1H, Hα-serine); 4.40 (m, 2H, CH_2-serine); 5.10 (AB, 2H, CH_2-benzyl); 7.20 - 7.40 (m, 5H, H-aromatic). ^{13}C NMR ($CDCl_3$): δ 27.0 - 28.0 (3x CH_3-tBu); 32.0 - 33.0 (2x CH_2S); 54.5 (Cα-serine); 62.2 (CH_2-OtBu); 66.0 - 70.0 (CH_2-serine, CH_2-benzyl, $PO\underline{C}H_2CH_2$); 73.3, 80.1, 82.9 (3x C_q-tBu); 128.0 -129.0 (C-aromatic); 135.8 (C_q-benzyl); 155.3 (NHC(O)); 168.2 (C(O)OtBu). **9b**: ^{31}P-NMR ($CDCl_3$): δ -1.30. ^1H-NMR ($CDCl_3$): δ 1.00 - 1.50 (3x s, 27H, CH_3-tBu), 2.65 - 3.00 (3x m, 6H, 2x CH_2S + CH_2CN); 3.60 (t, 2H, CH_2O-tBu), 4.05 - 4.50 (m, 7H, CH-serine, 3x CH_2OP), 5.50 (b, 1H, NH). **9c**: ^{31}P-NMR ($CDCl_3$): δ 0.30. ^{13}C-NMR ($CDCl_3$): δ 27.0 - 28.0 (3x CH_3-tBu); 32.9 (CH_2S, J_{PC} = 7.5 Hz); 55.4 (Cα-serine); 61.9 (CH_2O-tBu); 65.2 - 65.9 (CH_2-serine + $PO\underline{C}H_2CH_2$; J_{PC} = 5.6 Hz); 73.2, 79.3, 81.8 (3x C_q-tBu); 155.7 (NHC(O)); 169.3 (C(O)OtBu). **11**: ^1H-NMR ($CDCl_3$): δ 1.1 - 1.5 (3x s, 27H, CH_3-tBu), 3.0 - 3.2 (2x m, 4H, 2x $CH_2S(O)$), 3.9 (m, 2H, CH_2-OtBu), 4.1 (m, 1H, CH-serine), 4.1-4.4 (2x m, 4H, 2x CH_2OP). **12**: ^{31}P NMR (D_2O): δ 0.24 + 0.17 (mixture of diastereomers). Electrospray MS (positive ion mode) for: **15a** (calc. monoisot. mass: 2025.0), *m/z* 1013.6 $[MH_2]^{2+}$, 676.4 $[MH_3]^{3+}$; **15b** (calc. monoisot. mass: 2009.0), *m/z* 1005.5 $[MH_2]^{2+}$, 671.2 $[MH_3]^{3+}$, 105.1 $[HOCH_2CH_2SCH_2CH_2]^+$); **16a** (calc. monoisot. mass: 1116.5), *m/z* 1117.4 $[MH]^+$, 559.5 $[MH_2]^{2+}$; **16b** (calc. monoisot. mass: 1100.5), *m/z* 551.3 $[MH_2]^{2+}$, 105.1 $[HOCH_2CH_2SCH_2CH_2]^+$.
FPLC analysis of **15a/b** on PepRPC 5/5 column: Eluents (A) 0.1% TFA/H_2O; (B) 0.1% TFA in CH_3CN/H_2O (70/30, v/v). Flow rate 1.0 ml/min. Detection 214 nm. Gradient: 0 - 60% B in 20 min. Retention times: **15a**, 12.1 min.; **15b**, 13.1 min. For FPLC analysis of **16a/b**, see Figure 1.
18. Oxidation of thiodiglycol derivatives to sulfoxides is often accompanied with sulfone formation, especially when 3-chloroperbenzoic acid is used. For this reason no attempts were undertaken to perform phosphite and thioether oxidation in one single step. See, for instance, Black, R.M.; Brewster, K.; Harrison, J.M.; Stansfield, N. *Phosphorus, Sulphur and Silicon* **1992**, *71*, 31.
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