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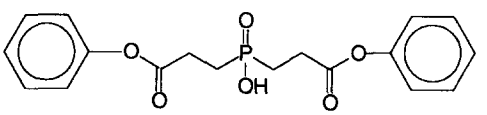
# A NOVEL CROSS-LINKING REAGENT FOR BIOMACROMOLECULAR MODIFICATION: BIS(PHENOXYCARBONYLETHYL)PHOSPHINIC ACID

Vishweshwar S. Bhadti, S. Prasad Peri, and Ramachandra S. Hosmane*

*Laboratory for Drug Design and Synthesis, Department of Chemistry and Biochemistry*
*University of Maryland Baltimore County, Baltimore, Maryland 21228 (U.S.A.)*

**Abstract**. Synthesis, properties, and reactions of the title reagent, bis(phenoxycarbonylethyl)phosphinic acid, are reported. The reagent's utility for biomacromolecular modification has been demonstrated by successfully cross-linking human hemoglobin. 

Cross-linking reagents have long played pivotal roles in probing structure and function of both proteins[1] and nucleic acids.[2] While natural cross-linkers such as mitomycin,[3] psoralens,[4] and anthramycins[5] have been extensively explored in nucleic acid chemistry, synthetic cross-linking reagents containing aldehyde,[6] imide,[7] ester,[8] azide,[9] imidate[10] or enol-ether[11] functionalities have dominated the protein field. However, these functional groups suffer from a variety of drawbacks, for example, while aldehydes readily form Schiff bases with amine nucleophiles of proteins and nucleic acids, the reactions are often reversible, requiring an additional reduction step[12] after the cross-link formation so as to stabilize the product. Ester and imide[13] groups, on the other hand, frequently show low reactivity under physiological conditions, whereas imidates[14] and enol-ethers[15] undergo facile hydrolysis. Azide couplings[16] are often photo-induced, and more often than not, yield multiple products. We report here the synthesis of a novel cross-linking reagent, bis(phenoxycarbonyl-ethyl)phosphinic acid (**1**), which contains the activated ester functionalities that are reactive enough toward amine nucleophiles, but are

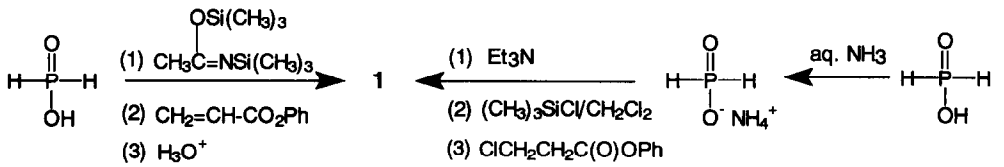


**1**

remarkably stable toward hydrolysis. Furthermore, the presence of an acidic phosphinic acid group affords facile conversion of **1** to its water-soluble alkali metal salts, thus enabling its use in the predominently aqueous medium of biological macromolecules. The reagent, a crystalline solid with an indefinite shelf-life, is easy and inexpensive to prepare from common starting materials, and with its one-step synthesis, can be conveniently produced on a large scale.

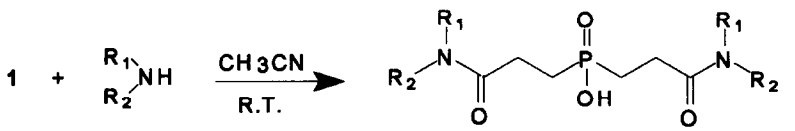
Reagent **1** was prepared by two different methods, using the known chemistry of phosphonous and phosphinic acids,[17] both commencing with hypophosphorous acid (Scheme 1). In the first method, an ice-cold solution of an equivalent of hypophosphorous acid in methylene chloride was sequentially treated with *N,O*-bis-trimethylsilylacetamide (3.6 equiv) and phenyl acrylate (2 equiv), and the reaction mixture was stirred at room temperature for 48 h. Acidification with 1N hydrochloric acid, extraction with chloroform, solvent removal, and recrystallization from ethanol gave **1** in 30% yield. The better-yielding (>60%) second method involved the initial transformation of hypophosphorous acid to its ammonium salt, followed by sequential treatments with triethylamine (3.5 equiv), chlorotrimethylsilane (3.5 equiv), and phenyl β-chloropropionate (2 equiv). The reagent is a colorless solid, mp 164-166 °C; $^1$H NMR ($CDCl_3$) δ 7.2 - 7.0 (m, 10 H, Ph), 4.67 (s, 1 H, OH), 3.0 - 2.9 (m, 4 H, $CH_2$), 2.28 - 2.02 (m, 4 H, $CH_2$); MS (FAB) *m/z* 363 ($MH^+$); Anal. Calcd. for $C_{18}H_{19}O_6P$: C, 59.67; H, 5.29. Found: C, 59.52; H; 5.29.

**Scheme 1**




The reactivity of **1** toward amine nucleophiles was assessed by reactions with a variety of primary and secondary amines, including *n*-butyl-, ethanol-, cyclohexyl-, phenyl-, and benzylamine and morpholine, at room temperature, using acetonitrile as the solvent (Scheme 2). The bis-amide products **2a-2f** were characterized by spectral and microanalytical data.[18]

**Scheme 2**



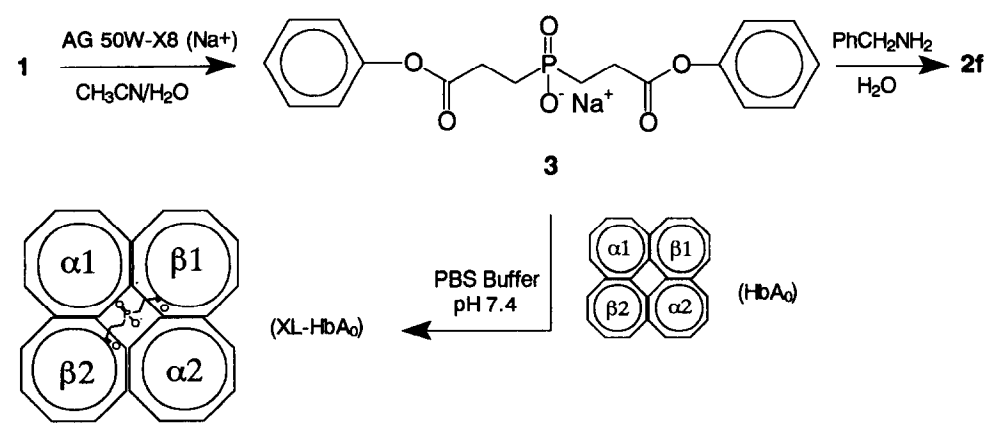

**2**

a; $R_1$=H, $R_2$=$(CH_2)_3CH_3$
b; $R_1$=H, $R_2$=$(CH_2)_2OH$
c; $R_1$,$R_2$=morpholin(*N*-yl)
d; $R_1$=H, $R_2$=cyclohexyl
e; $R_1$=H, $R_2$=phenyl
f; $R_1$=H, $R_2$=benzyl

In order to assess its suitability for aqueous systems, reagent **1** was further converted to its sodium salt (**3**) (Scheme 3) by passing a solution of **1** in aqueous acetonitrile through a column packed with the resin AG 50W-X8 ($Na^+$ form), and eluting with water. The product was reacted with benzylamine as before, but using water as the solvent, which gave **2f** in a comparable yield of 73%. No products of hydrolysis were detected at the ambient temperature in which the reaction was carried out. Furthermore, there was no change in the UV absorbance of reagent **3** in the pH 7.5-8.0 range (PBS buffer) even after 24 hours.

Finally, the sodium salt **3** was reacted with human hemoglobin ($HBA_0$) under oxygenated conditions in PBS buffer (pH 7.4) at room temperature. A typical experimental procedure involved

**Scheme 3**



incubation of 100 μL of 0.77 mM $HbA_0$ with 15 μL of 500 mM reagent in a total buffer volume of 150 μL for 60 minutes. Isoelectric focusing (IEF) analysis of the reaction mixture indicated that the reaction had taken place, by revealing modified protein that had the pI in the more acidic region, *i.e.* below the pI of the unmodified $HbA_0$. This observation is consistent with neutralization of some of the positive charges present on the protein upon covalent cross-linking. However, since charge neutralization could also result from mere electrostatic interaction of the positive charge of protein with the phosphinate anion of the cross-linker, the reaction mixture was further subjected to SDS-PAGE, HPLC and FPLC analyses. Under the drastic denaturing conditions of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), purely ionic interactions would be overcome, and only the covalent cross-links are detectable. The SDS-PAGE analysis of the reaction mixture (Figure 1) clearly indicated the presence of covalent cross-links due to the presence of bands corresponding to dimers of the monomer units with a molecular weight of ~ 32 kDa. The HPLC chromatogram (Vydac $C_4$ protein column, pore size 300 μ, particle size 5 μ) of the diluted reaction mixture in 0.1% trifluoroacetic acid (TFA), employing a gradient of 35-50% acetonitrile-water with 0.1% TFA as an eluting solvent with a flow rate of 1 mL/min, revealed the presence of two newly cross-linked hemoglobins with retention times of 46 and 51.5 min, along with the unreacted $HbA_0$ with retention times of 38 and 41 min, corresponding to the β and α chains, respectively. Furthermore, since the peak integration of only the β chains of the unreacted $HbA_0$ was considerably reduced as compared to the reference standard, the chemical modification seems to lie predominently in the β chains. The FPLC analysis (two Pharmacia Superose-12 columns, connected in series, each 10x300 mm, particle size 10 microns) of the reaction mixture under dissociating conditions, employing either PBS or 50 mM bis-tris buffer (pH 7.4) in the presence of 100 mM $MgCl_2$ for elution, corroborated the above finding, by revealing a distinct peak that corresponded to cross-links between the like subunits of $HbA_0$ (*e.g.* ββ) that eluted earlier (retention time 56 min) than the peak that corresponded to $HbA_0$ that is either cross-linked between the unlike subunits (αβ) or is uncross-linked (retention time 59.9 min).

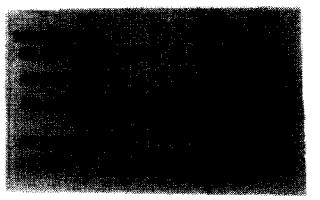


Fig. 1: SDS-PAGE Analysis.
Lanes 1 & 6: M. W. Markers;
Lane 2: HbA Ref. (3.0 μg);
Lane 3: XLHbA (1.0 μg);
Lane 4: XLHbA (2.0 μg);
Lane 5: XLHbA (3.0 μg).

In light of the keen interest in hemoglobin cross-linking in search of an efficacious and pathogen-free blood substitute for emergency transfusion,[19] heightened by the current epidemic of blood-borne diseases such as AIDS and hepatitis, the present work carries a timely significance.

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18. The spectral and microanalytical data for compounds **2a-2f** are as follows:
**2a**; $R_1$=H, $R_2$=$(CH_2)_3CH_3$; mp 126-128 °C; yield 90%; $^1$H NMR (DMSO-$d_6$) δ 8.42 (t, 2 H, *J* = 4.8 Hz, NH, exchangeable with $D_2O$), 3.37 (br s, 1 H, OH), 2.99 (q, 4 H, *J* = 6.4 Hz, $CH_2$), 2.66 (t, 4 H, *J* = 7.5 Hz, $CH_2$), 2.23-2.14 (m, 4 H, $CH_2$), 1.52-1.19 (m, 8 H, $CH_2$), 0.84 (q, 6 H, *J* = 6.9 Hz, $CH_3$); Anal. Calcd. for $C_{14}H_{29}N_2O_4P$: C, 52.49; H, 9.12; N, 8.74. Found: C, 52.57; H, 9.11; N, 8.79. **2b**; $R_1$=H, $R_2$=$(CH_2)_2OH$; mp 140-142 °C; yield 86%; $^1$H NMR (DMSO-$d_6$) δ 7.85 (t, 2 H, *J* = 4.8 Hz, NH, exchangeable with $D_2O$), 4.02 (br s, 1 H, OH), 3.49 (t, 4 H, *J* = 5.7 Hz, $CH_2$), 3.19 (q, 4 H, *J* = 5.7 Hz, $CH_2$), 2.46-2.37 (m, 4 H, $CH_2$), 1.95-1.85 (m, 4 H, $CH_2$); Anal. Calcd. for $C_{10}H_{21}N_2O_6P$: C, 40.54; H, 7.14; N, 9.46. Found: C, 40.28; H, 7.15; N, 9.25. **2c**; $R_1R_2$= morpholin(*N*-yl); mp 148-150 °C; yield 76%; $^1$H NMR (DMSO-$d_6$) δ 6.36 (br s, 1 H, OH), 3.67-3.61 (m, 16 H, $CH_2$), 2.75-2.66 (m, 4 H, $CH_2$), 2.11-2.07 (m, 4 H, $CH_2$); Anal. Calcd. for $C_{14}H_{25}N_2O_6P.0.5\ H_2O$: C, 47.06; H, 7.33; N, 7.84. Found: C, 47.37; H, 7.13; N, 7.68. **2d**; $R_1$=H, $R_2$=cyclohexyl; mp 202-204 °C; yield 78%; $^1$H NMR (DMSO-$d_6$) δ 7.79 (d, 2 H, *J* = 7.8 Hz, NH, exchangeable with $D_2O$), 4.15 (br s, 1 H, OH), 3.50-3.49 (m, 2 H, CH), 2.37-2.31 (m, 4 H, $CH_2$), 1.81-1.5 (m, 14 H), 1.27-1.17 (m, 10 H); Anal. Calcd. for $C_{18}H_{33}N_2O_4P$: C, 58.05; H, 8.93; N, 7.52. Found: C, 58.12; H, 8.94; N, 7.44. **2e**; $R_1$=H, $R_2$=phenyl; mp 215-217 °C; yield 90%; $^1$H NMR (DMSO-$d_6$) δ 10.0 (s, 2 H, NH, exchangeable with $D_2O$), 7.57-7.00 (m, 10 H, Ar-H), 4.10 (br s, 1 H, OH), 2.63-2.51 (m, 4 H, $CH_2$), 2.01-1.91 (m, 4 H, $CH_2$); Anal. Calcd. for $C_{18}H_{21}N_2O_4P$: C, 60.00; H, 5.87; N, 7.77. Found: C, 60.07; H, 5.91; N, 7.70. **2f**; $R_1$=H, $R_2$=benzyl; mp 229-231 °C; yield 70%; $^1$H NMR (DMSO-$d_6$) δ 8.45 (t, *J* = 5.7 Hz, 2 H, NH, exchangeable with $D_2O$), 7.32-7.22 (m, 10 H, Ar-H), 4.25 (d, *J* = 6.0 Hz, 4 H, benzyl $CH_2$), 2.40-2.31 (m, 4 H, $CH_2$), 1.83-1.76 (m, 4 H, $CH_2$); Anal. Calcd. for $C_{20}H_{25}N_2O_4P$: C, 61.49; H, 6.49; N, 7.21. Found: C, 61.77; H, 6.49; N, 7.15.
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