Pergamon

Tetrahedron Letters, Vol. 35, No. 34, pp. 6191-6194, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)01299-7

A General Method for Coupling Unprotected Peptides to Bromoacetamido Porphyrin Templates

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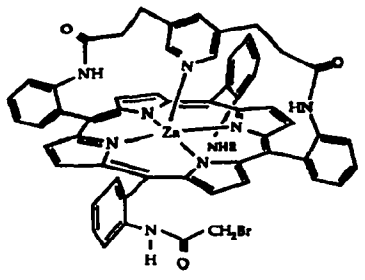
Abstract: An N-terminal cysteine is used to displace bromide from a bromoacetylated porphyrin to yield a thioether linkage between the peptide and the template. Unlike amide coupling reactions, this approach should be compatible with any peptide sequence provided there is only a single cysteine.

An important test of our understanding of the physical interactions that drive the folding process and stabilize the final structure is to design novel protein sequences which self-assemble into well-defined three-dimensional structures.[1] A variety of proteins have been designed including a family of 'minimalist' four-helix bundles[2] and bundles that tightly and specifically bind one[3] or several[4] Fe-protoporphyrin IX molecules. In a logical extension of this work, we aspired to attach these proteins to Zn^{2+}-tetraphenyl porphyrin templates in order to construct synthetic complexes suitable for studies of photo-induced electron transfer.[5]

Porphyrin derivatives have served as templates for 'TASPs' (template-assembled synthetic proteins[6]) with aniline hydroxylase[7] and proton channel[8] activity. In these studies, peptides were coupled to porphyrin derivatives via amide bonds using standard amide coupling reagents. This approach requires either the presence of protecting groups on lysine and glutamine sidechains, or restricting the sequence to non-reactive residues. A recently reported method circumvents some of these synthetic limitations and involves the formation of a thioester bond between the peptide and the template.[9] However, we were concerned about the hydrolytic stability of thioester linkages, and so we have devised a novel approach to link unprotected peptides to a porphyrin via stable thioether bonds.

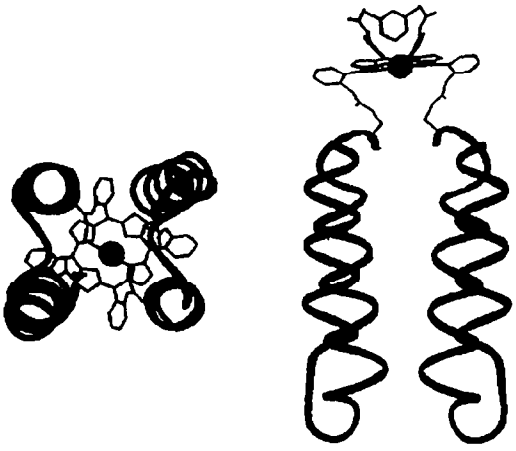


(1)

The thioether bond is formed by a cysteine thiol displacing the bromide of α,α-5, 15-(2,2'-[3,3'(pyridine-3,5-diyl)-dipropionamido] diphenyl):β,β-10,20-bis(*o*-(2-bromoacetamido)phenyl)-porphyrinatozinc (II) (1). Phenyl substitutions at the *meso* positions protect the porphyrin ring from oxidation, and peptide attachment to the *ortho* position on the phenyl rings provides favorable interhelical spacing. Only the β-positions of (1) are available as peptide attachment sites since the α positions are coupled to a pyridyl strap straddling the 'upper' porphyrin face. The pyridyl nitrogen ligates the zinc[10] resulting in a porphyrin with a tightly bound metal ion. In addition, the strap should reduce

the possibility of the porphyrin aggregating. Cys-α_2, the parent peptide of one of our heme-binding four-helix bundles[3] was used to determine the overall synthetic approach for the coupling reaction. We here describe an optimized synthesis of the porphyrin and conditions for its covalent attachment to two copies of unprotected Cys-α_2. The resulting assembly, α,α-5,15-pyridine strap:β,β-10,20-bis(*o*-(cys-α_2)-2-thioacetylamidophenyl porphyrin (**2**) should fold with two pairs of anti-parallel α-helices projecting from one face of the tetraphenyl porphyrin framework.

The strapped porphyrin was prepared by coupling a single molecule of pyridine-3,5-bis(propionic acid) (**3**) to Zn-$\alpha,\beta,\alpha,\beta$–tetrakis-(*o*-amino)tetraphenyl porphyrin (**4**) using the mixed anhydride method. The resulting diamine was reacted with bromoacetic acid and dicyclohexyl-carbodiimide providing the bis-bromoacetylated derivative of (**1**). A variety of conditions were then examined for the attachment of a Cys-containing test peptide representing the first eleven residues of Cys-α_2 to (**1**); these conditions included pH in the range 7.8-9.2, temperatures ranging from 21-50 °C, and a variety of organic solvent and organic/aqueous buffer solvents. Predominant formation of the bis-adduct was obtained using 60:40 ethanol:50 mM HEPES[11] buffer brought to pH 8.0 with N-methylmorpholine and stirring the reactants overnight at 45 °C under an inert atmosphere. Using these conditions, the 36-residue helix-loop-helix Cys-α_2 peptide was coupled to (**1**), resulting in the synthesis of the bis-adduct in 24% isolated yield after chromatography by HPLC. The approach described here for linking one peptide molecule per bromoacetyl site on a bromoacetamido template should be compatible with any mono-Cys peptide sequence.



(2) top view (2) side view

Experimental Procedures[12]

(a) Zn-α,β,α,β–tetrakis-(o-amino)tetraphenyl porphyrin (4) The porphyrin (**4**) was synthesized essentially as described by Collman.[13] The desired atropoisomer was obtained by silica gel chromatography[14] and the free base porphyrin was metalated as described previously.[15] (^{1}H NMR ($CDCl_3$) δ 8.52 (8H,s), 7.67 (4H,d), 7.35 (4H,t), 7.08 (4H,t), 6.26 (4H,d). UV ($CHCl_3$) 426(λ_{max}) 554 nm.

(b) pyridine-3,5-bis(propionic acid) (3) Pyridine-3,5-dicarboxylic acid (5 g, 29.9 mmol) was stirred in 50 mL toluene with 10 mL thionyl chloride and heated under reflux for 1 h. Excess thionyl chloride was removed by distillation and the resulting pyridine-3,5-diacid chloride (**5**) was dissolved in dry THF (25 mL). To (**5**) at -78 °C under an inert atmosphere, lithium tri-*tert*-butoxyaluminohydride (1.0 M in THF, 66 mL) was added slowly, stirred for 1 h and then quenched with a minimum amount of H_2O. The resulting solid was filtered, rinsed with ethanol and dried in vacuo to yield pyridine-3,5-dialdehyde (**6**) as the main product (1.5 g, 11 mmol, 37%).[16] A portion of (**6**) (0.945 g, 7 mmol) was placed in a 25 mL flask and malonic acid (2.92 g., 28 mmol), pyridine (10 mL), and piperidine (500 μL) were added. The reaction was heated at 105 °C for 10 h. The solvent was removed by concentration in vacuo and the white solid was rinsed with H_2O and filtered. ^{1}H NMR

spectroscopy confirmed the compound as pyridine-3,5-bis(propenoic acid) (7) (0.875 g, 4 mmol, 57%).[17] This compound (0.5 g, 2.3 mmol) was hydrogenated in ethanol (20 mL) and HCl (1 mL) in the presence of 10% Pd/C (700 mg) under an H_2 atmosphere for 5 h at 50 °C. The reaction was filtered and the filtrate concentrated to dryness. The solid was recrystallized from methanol (0.388 g, 1.7 mmol, 76%) and identified as (3). ^1H NMR (D_2O) δ 8.43 (2H,s), 8.30 (1H,s), 2.98 (4H,t), 2.68 (4H,t).

(c)α,α-5,15-(2,2'-[3,3'(pyridine-3,5-diyl)-dipropionamido]diphenyl):β,β-10,20-bis(o-aminophenyl)porphyrinatozinc(II) (8) The porphyrin strap (3) (27 mg, 120 μmol) was dissolved in CH_2Cl_2 (12 mL) and triethylamine (52 μL) and the solution was cooled to 0 °C. Isobutyl chloroformate (32 μL, 240 μmol) was added and the mixture was stirred 15 min. In a separate flask, (4) (55 mg, 80 μmol) was dissolved in CH_2Cl_2 (25 mL), triethylamine (120 μL), and dimethylaminopyridine (10 mg). The anhydride solution was added dropwise to the solution of (4) and the mixture was stirred o.n. at 21 °C.[18] The reaction mixture was concentrated in vacuo and (8) was purified by preparative TLC to yield 29 mg (31 μmol, 40%). ^1H NMR ($CDCl_3$)[10] δ 8.85 (8H,dd), 8.61 (2H,m), 8.03 (2H,d), 7.84 (4H,m), 7.64 (2H,m), 7.52 (2H,t), 7.13 (2H,t), 6.97 (2H,d), 6.78 (2H,s), 5.75 (1H,s), 3.20 (4H,br), 2.16 (2H,s). Fast atom bombardment mass spectrometry: (m/z) 925 (M^+, 60), 621 (13), 613 (100), 603 (20), 597 (55), 595 (25). UV (CH_2Cl_2) 432 (λ_{max}), 562, 604 nm.

(d)α,α-5,15-(2,2'-[3,3'(pyridine-3,5-diyl)-dipropionamido]diphenyl):β,β-10,20-bis(o-(2-bromoacetamido) phenyl)-porphyrinatozinc(II) (1) Bromoacetic acid (1.25 mg, 8.9 μmol) in 17 μL CH_2Cl_2 and dicyclohexyl carbodiimide (1.84 mg, 8.9 μmol) in 56 μL CH_2Cl_2 were added to 1.04 mg (1.12 μmol) solid porphyrin (8). The mixture was stirred o.n. at 21 °C in the absence of light in a tightly sealed conical 0.3 mL glass vial. The product was purified by silica gel chromatography (1 x 15 cm column: 100 mL 4% methanol:96% CH_2Cl_2) and was dried under N_2 to provide 0.93 mg (71%) of a black solid identified as (1) by electrospray mass spectrometry ($M+H^+$ 1165.16 expected, 1165.2 ± 1.4 found). Analytical HPLC indicated that (1) (major peak eluting at 47% acetonitrile) was approximately 95% pure.

(e) α,α-5,15-pyridine strap:β,β-10,20-bis(o-(cys-α2)-2-thioacetylamidophenyl porphyrin (2) Reduced Cys-α2 (11 mg, 2.6 μmol) in 150 μL of 60:40 ethanol:50 mM HEPES (brought to pH 8.0 with N-methylmorpholine) was added to 0.5 mg (0.43 μmol) solid (1) and stirred under Ar and in the absence of light o.n. at 45 °C. The products were purified by HPLC using a 1.2 cm x 25 cm Vydac C_4 column. The monoadduct ($M+H^+$ = 5323 ± 0.93, 16% yield) eluted at 57% buffer B (90% acetonitrile, 10% water, 0.1%TFA) whereas the bisadduct (2) ($M+H^+$ = 9438 ± 2.0, 24% yield) eluted at 61% buffer B.

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11. *Abbreviations:* HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); HPLC, high dimethyloxyphenoxyl)-valeric acid; o.n., over night; TLC, thin layer chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran.
12. *Materials:* The peptide Cys-α_2 was synthesized by Fmoc chemistry on PAL resin as described previously.[3] Chloroform and methylene chloride were freshly distilled over calcium hydride and tetrahydrofuran was distilled over sodium/benzophenone. All other chemicals were of the highest available grade and were used without further purification. Preparative TLC was performed on Analtech G 1000 micron plates, and flash chromatography using silica gel 60 from EM Science. Proton NMR spectra were taken on a JEOL GSX 270 (270 MHz) spectrometer and chemical shifts are reported relative to residual solvent resonances ($CDCl_3$ δ 7.24, D_2O δ 4.67). UV spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrometer. Analytical HPLC was performed on a Hewlett Packard 1090 liquid chromatograph using a Vydac C_4 column and a linear acetonitrile/water gradient, and preparative HPLC was performed on a Rainin; the eluent was monitored at 415 nm. Amino acid analysis was performed on a Beckman 6300 high-performance analyzer, electrospray mass spectrometry on a Fisions Instruments Trio-2000 mass spectrometer and fast atom bombardment mass spectrometry on a Kratos MS50 RFA mass spectrometer.
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19. This work was supported by grants from The Office of Naval Research and the National Institutes of Health (GM 36298).

(*Received in USA* 6 *May* 1994; *revised* 17 *June* 1994; *accepted* 30 *June* 1994)