Pergamon

Tetrahedron Letters 39 (1998) 1665–1668

TETRAHEDRON LETTERS

Synthesis and Photochemistry of Two Cleavable Heterobifunctional Benzophenone Protein Crosslinkers

John E. Oatis, Jr and Daniel R. Knapp*

Department of Pharmacology, Medical University Of South Carolina, Charleston, South Carolina 29425

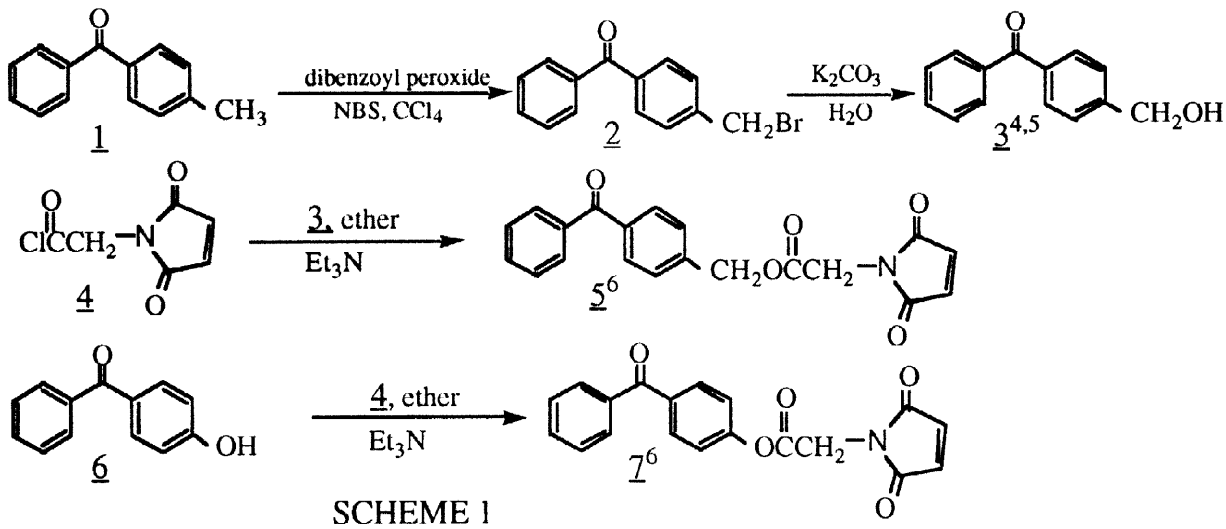
Received 27 June 1997; accepted 17 December 1997

Abstract: Two new heterobifunctional protein crosslinkers were synthesized. These reagents contain the cysteine reactive maleimido group connected via a cleavable ester linkage to the benzophenone photoactivatable group.

© 1998 Elsevier Science Ltd. All rights reserved.

Chemical, photochemical, and heterobifunctional chemical-photochemical crosslinking agents have been employed extensively to probe the structure and function of enzymes and receptor proteins. While many of the photoreactive reagents contain azido, diazo or diazirine groups as photophores, more recently benzophenone[1] photoprobes has been extensively used to study nucleotide, receptor and enzyme binding sites; combined with a chemically reactive group, the benzophenone containing reagents have also been employed as crosslinkers to investigate intramolecular as well as protein-protein interactions. The benzophenone photoprobe can be superior to other photoactivatable groups since the benzophenone group is chemically more stable, and the benzophenone is photoactivated at 350-360 nm avoiding protein damaging shorter wavelengths. Upon photoactivation, the reactive triplet state inserts into C-H bonds, even in the presence of water or other nucleophiles; but in the absence of an abstractable proton, the triplet will relax to the ground state, thus allowing many excitation-relaxation cycles. In spite of these advantages, only a few heterobifunctional benzophenone reagents have been reported, and those described have a significant disadvantage in that none are cleavable. In a protein crosslinking study with these reagents, sequencing the resulting linked peptides to determine the exact site of incorporation of the photoprobe can be difficult. In this paper we describe the synthesis, photochemistry and hydrolysis of two cleavable benzophenone crosslinking reagents that can be selectively introduced onto a cysteine residue and, after photoactivation, can be cleaved under mild conditions to leave only the benzophenone remnant as a tag at the labelled site.

As depicted in Scheme 1, 4-bromomethylbenzophenone **2**, synthesized via the method of Houlihan and Nadelson,[2] was hydrolyzed with potassium carbonate to yield 4-hydroxylmethyl benzophenone **3**. Esterification of both **3** and 4-hydroxybenzophenone **6** with maleimidoacetyl chloride **4**, prepared via the method of Paul, *et al*,[3] yielded the desired crosslinkers **5** and **7**.

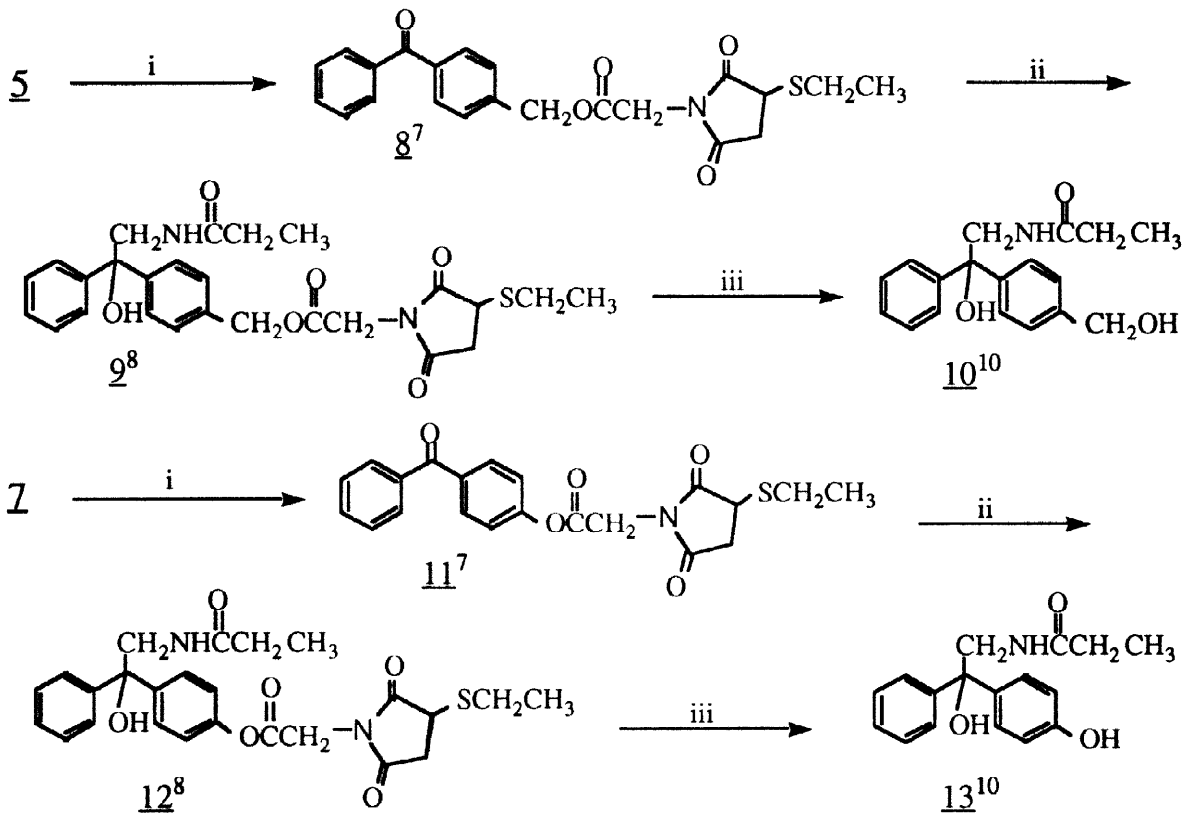


SCHEME 1

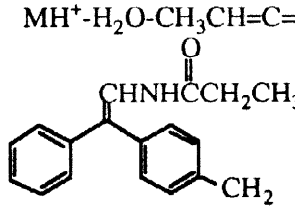
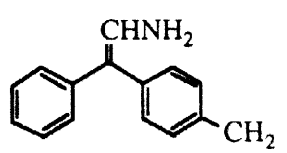
0040-4039/98/$19.00 © 1998 Elsevier Science Ltd. All rights reserved.

PII: S0040-4039(98)00016-1

To assess the abilities of the crosslinkers to photoinsert, **5** and **7** were first converted to the ethyl sulfides **8** (Scheme 2) and then were photolyzed at 350 nm, 4°C for 40 min in a protein mimicking system, neat N-methyl propionamide. In this system, LC-MS[4] showed that under the same conditions both **8** and **11** photoinsert into N-methyl propionamide. As depicted in Figure 1, photoproducts **9** and **11** have intense



Scheme 2. i. 1M HEPES (pH8.0) - CH_3CN(1:1), CH_3CH_2SH ii. neat N-methylpropionamide, hv, 350nm, 4°C, iii. 4.32 M $NH_2OH.HCl$ (pH 8.6) - n-propanol(1:1).

A	m/z	ion
	499	MH^+
	481	MH^+-H_2O
	425	$MH^+-H_2O-CH_3CH=C=O$
	264	
	208	

B	m/z	ion
	485	MH^+
	467	MH^+-H_2O
	411	$MH^+-H_2O-CH_3CH=C=O$

Figure 1. Selected fragment ions obtained in the MS-MS spectra of **9**(panel A)and **12**(panel B).

MH+ ions at m/z 499 and 485 respectively; when these molecular ions are fragmented the loss of H_2O predominates. As shown in Figure 1A, **9** gives additional fragments at m/z 425 and 208, suggesting that insertion occurs at the N-methyl, since no fragment ions indicative of other insertion sites were observed. Compound **11** gives a similar fragmentation pattern (Figure 1B) with a fragment ion at m/z 411 indicative of N-methyl insertion. NMR spectra substantiate these conclusions.[8] Insertion of the benzophenone photoproduct into a C-H bond adjacent to a heteroatom has previously been reported.[1,9]

To demonstrate cleavage of the photolyzed crosslinkers, **9** and **12** were treated at room temp with a 154 fold excess of hydroxylamine in 50% aqueous (pH 8.5)-n-propanol. For **9**, complete cleavage to **10** requires 24 hrs, whereas **12** was completely hydrolyzed to **13** in only 6 hrs. Product **10**, appears to be stable, but product **13** appears prone to decompose as indicated by extraneous NMR peaks in spite of its showing a single HPLC peak. This is likely due to the propensity of **13** to dehydrate to an unstable enamide. This factor may limit the utility of **10** as a protein crosslinking agent.

In summary, we have synthesized two new heterobifunctional protein crosslinkers and showed that when photolyzed at 350 nm, they photoinsert into a protein mimicking solvent, neat N-methylpropionamide, yielding photoproducts which can be cleaved.

ACKNOWLEDGEMENT

Spectral data were obtained using the MUSC Nuclear Magnetic Resonance and Mass Spectrometry Research Resource Facilities. We acknowledge the assistance of Dr. Kuruppu Dharmasiri for acquiring the high resolution mass spectra. This work was supported by Grant EY08239 from the National Institutes of Health.

REFERENCES AND NOTES

1. Dorman, G.; Prestwich, G. L. *Biochemistry* **1994**, 33, 5661-5673.
2. Houlihan, W. J.; Nadelson, J. US Patent 3,927,079 (Chem. Abstr.**1976**, 84, P7392v).
3. Paul, L.; A. Dittmar, A.; Rusch, C. *Chem. Ber.* **1967**, 100, 2757-2761.
4. NMR spectra were acquired on a Varian VXR 400 spectrometer. FAB MS spectra were obtained on JEOL HX110/HX110 mass spectrometer. LC-MS data were obtained on a Finnigan LCQ mass spectrometer using an atmospheric pressure chemical ionization (APCI) source and interfaced to an HP1100 series liquid chromatograph employing a 0.46X25cm Vydac C-18 column. The components were eluted with 98% A for 3 min, then a gradient of 98% A to 2% A over 40 min with a flow rate of 0.7 mL/min. Solvent A: 0.1% TFA-H_2O and solvent B:0.085% TFA in CH_3CN.
5. 4-hydroxymethyl benzophenone **3:** A solution of **2** (6.8 g, 25 mmol), and K_2CO_3 (13.8 g, 100 mmol) in 110 mL H_2O was refluxed for 18 hrs. After cooling to room temp, the product was extracted with 2 X 100 ml $CHCl_3$, the organic solution was dried ($MgSO_4$), and the solvent was removed at reduced pressure to yield 5.2 g of a yellow oil. The product was purified by HPLC (2.54 X 50 cm silica gel column) eluting with $CHCl_3$. The yield was 3.24g mp 59-61 (61% from **1**) NMR ($CDCl_3$) δ7.76 (m, 4, aromatic), 7.58(m, 1, aromatic), 7.46 (m, 4, aromatic), 4.77(s, 2, CH_2); HRMS: $C_{14}H_{13}O_2$ requires 213.0916 found 213.0904.
6. Representative esterification: 4-Benzophenonemethyl maleimidoacetate **5:** To an ice cold solution of **3** (740 mg, 3.49 mmol) and **4** (2.0 g, 11.5 mmol) dissolved in anhydrous ether was added over 15 min Et_3N (2.5 mL, 1.82 g,17.9 mmol). After the addition was complete, the reaction was stirred for 15 min at 0°C, then stirred at room temperature for 18 hrs. The ether was removed with reduced pressure, the residue triturated with H_2O, then dried in vacuo over P_2O_5. The product was purified by passing through a short column of silica gel, eluting with CH_2Cl_2. The yield was 415 mg(34%). **5:** mp 107-109, NMR ($CDCl_3$) δ7.81 (m, 4, aromatic), 7.61 (m, 1, aromatic), 7.49 (m, 2, aromatic), 7.44 (m, 2,aromatic), 6.81 (s, 2, olefin), 5.26 (s, 2, OCH_2), 4.37 (s, 2,NCH_2); HRMS: $C_{20}H_{15}NO_5$ requires 350.1028 found 350.1027. **7:** (33 %) mp 138-139, NMR ($CDCl_3$) δ7.85 (m, 2, aromatic), 7.79(m, 2, aromatic), 7.60 (m, 1, aromatic), 7.49 (m, 2, aromatic),7.26 (m, 2, aromatic) 6.85 (s, 2, olefin), 4.68 (s, 2, NCH_2); HRMS: $C_{19}H_{13}NO_5$ requires

336.0872 found 336.0892.

7. Representative reaction with ethanethiol: 4-Benzophenonemethyl 3'-ethylthiosuccinimidoacetate **8:** To a solution of **5** (44.8 mg, 0.13 mmol) dissolved in 500 µL 1M HEPES(pH 8.0)-CH_3CN (4:1) was added ethanethiol (20 µL,16.8 mg, 0.27 mmol) and the reaction stirred at room temperature for 3 hrs. After removing the solvents under vacuum, the product was purified by passing it through a short column of silica gel, eluting with CH_2Cl_2 to give a colorless oil. The yield was 30 mg (57%).**8:** NMR($CDCl_3$) δ7.81 (m, 4, aromatic), 7.61 (m, 1, aromatic), 7.50 (m, 2, aromatic), 7.46 (m, 2, aromatic), 5.27 (d, J=12, 8 Hz, 1, CH_2O), 5.26 (d, J=12, 8 Hz, 1, CH_2O), 4.36 (s, 2, NCH_2), 3.85 (dd, J=3.8 Hz, J=9, 2 Hz, 1, CHS), 3.25 (dd, J=9.2 Hz, J=18.8 Hz, 1, C$\underline{H}_2$CHS), 2.92 (qd, J=7.4 Hz, J=12.5 Hz, 1, SCH2), 2.78 (qd, J=7.4 Hz, J=12.5 Hz, 1, SCH_2), 2.64 (dd, J=3.8 Hz, J=18.8 Hz, 1, C$\underline{H}_2$CHS, 1.32 (t, J=7.4 Hz, 3, CH_3), HRMS: $C_{22}H_{21}NO_5S$ requires 412.1219 found 412.1209. **11:** (51%) NMR ($CDCl_3$) δ7.86 (m, 2, aromatic), 7.79 (m , 2, aromatic), 7.60 (m, 1, aromatic), 7.50 (m, 2, aromatic), 7.26 (m, 2, aromatic), 4.56 (s, 2, NCH_2), 3.88 (dd, J=9.2 Hz, J=3.8 Hz, 1,CHS), 3.29 (dd, J=9.2 Hz, J=18.9 Hz), 2.93 (qd, J=7.4 Hz, J=12.5 Hz, 1, SCH_2), 2.79 (qd, J=7.4 Hz, J=12.5 Hz, 1, SCH_2), 2.68 (dd, J=3.8 Hz, J= 18.9 Hz), 1.32(t, J=7.4 Hz,3,CH_3), HRMS: $C_{21}H_{19}NO_5S$ requires 398.1062 found 398.1028.
8. Representative photolysis in N-methylpropionamide: **8** (9.4 µmol) dissolved in 750 µL N-methyl propionamide, deoxygenated by argon, was cooled to 4°C, and photolyzed under argon at 350 nm for 40 min. The solvent was removed under vacuum and the residue purified by HPLC. **9:**(50 %). NMR ($CDCl_3$) δ7.43 (m, 3, aromatic), 7.23-7.38 (m, 6, aromatic), 5.73 (bt, J=5.Hz, 1, NH), 5.16 (dd,J=12.8 Hz,1, OCH_2), 5.15 (d, J=12, 8 Hz, 1, CH_2O), 4.30 (s, 2, $OCCH_2N$), 4.08 (d, 2, J=5.9 Hz, C$\underline{H}_2$NH), 3.83(dd, J=3.8 Hz, J=9.2 Hz, 1, CHS), 3.24 (dd, J=9.2 Hz, J=18.8 Hz, 1, C$\underline{H}_2$CHS), 2.90 (qd, J=7.4 Hz, J=12.5 Hz, 1, SCH_2), 2.77 (qd, J=7.4 Hz, J=12.4 Hz, 1, SCH_2), 2.64 (dd, J=3.8 Hz, J=18.8 Hz, 1, C$\underline{H}_2$CHS), 2.14 (q, J=7.6 Hz, $COCH_2$), 1.30 (t, J=7.4Hz, 3, $SCH_2C\underline{H}_3$), 1.05 (t, J=7.6 Hz, $COCH_2C\underline{H}_3$), HRMS: $C_{26}H_{30}N_2O_6S$ requires 499.1903 found 499.1876. **12:** (40%). NMR ($CDCl_3$) δ7.45 (m, 2, aromatic), 7.42 (m , 2, aromatic), 7.35(m, 2, aromatic), 7.27 (m, 1, aromatic), 7.09, 2, aromatic), 5.74 (bt, 1, J=6.0 Hz, NH), 4.51 (s, 2, $COCH_2N$), 4.06 (d, J=6.0 Hz, 2, C$\underline{H}_2$NH), 3.86 (dd, J=3.7Hz, J=9.2 Hz, 1, CHS), 3.27 (dd, J=9.2 Hz, J=18.8 Hz, 1, C$\underline{H}_2$CHS), 2.93 (qd, J=7.4 Hz, J=12.5 Hz, 1, SCH_2), 2.79 (qd, J=7.4 Hz, J=12.5 Hz, 1,SCH_2), 2.68 (dd, J=3.8 Hz, J=18.9 Hz), 2.14 (q, 2, J=7.6 Hz, $COCH_2$), 1.32 (t, J=7.4 Hz, 3,$SCH_2C\underline{H}_3$), 1.05 (t, J=7.6 Hz,$OCCH_2C\underline{H}_3$). HRMS: $C_{25}H_{26}N_2O_5S$ requires 467.1641 found 467.1682.
9. Kagnet, R.; Leeman, S. E.; Krause, J. E.; Costello, C. E.; Boyd, N. D.. *J. Biol. Chem.* **1996**, 271, 25797-25800.
10. Representative hydrolysis: **10** (4.8 µmole) dissolved in 200 µL n-propanol and 200 µL aqueous 4.32 M NH_2OH·HCl (pH8.6) (207 µmol) was stirred at ambient temperature. After 24 hrs starting **10** was consumed, and the reaction was quenched by the addition of 600 mL 1M pH7.4 Tris. The solvents were removed under vacuum, then purified by HPLC. **10** (quantitative): NMR ($CDCl_3$) δ7.45 (m, 2, aromatic), 7.43 (m , 2, aromatic), 7.36(m, 2, aromatic), 7.34 (m, 2, aromatic), 5.71(bt, 1, NH), 4.69 (s, 2, C$\underline{H}_2$OH), 4.09 (d, J=5.9 Hz, 2, $COCH_2N$), 2.14 (q,J=7.6 Hz, 2, $COCH_2$), 1.05 (t, J=7.6 Hz, 3, $COCH_2C\underline{H}_3$), HRMS: $C_{18}H_{21}NO_3$ requires 300.1600 found 300.1602.**13**: HRMS (MH^+-H_2O) $C_{17}H_{17}NO_2$ requires 268.1338 found 268.1340.