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Synthesis of Activated Disulfide Adducts Containing a 4-Diazocyclohexa-2,5-dienone Precursor for Photoaffinity Labelling

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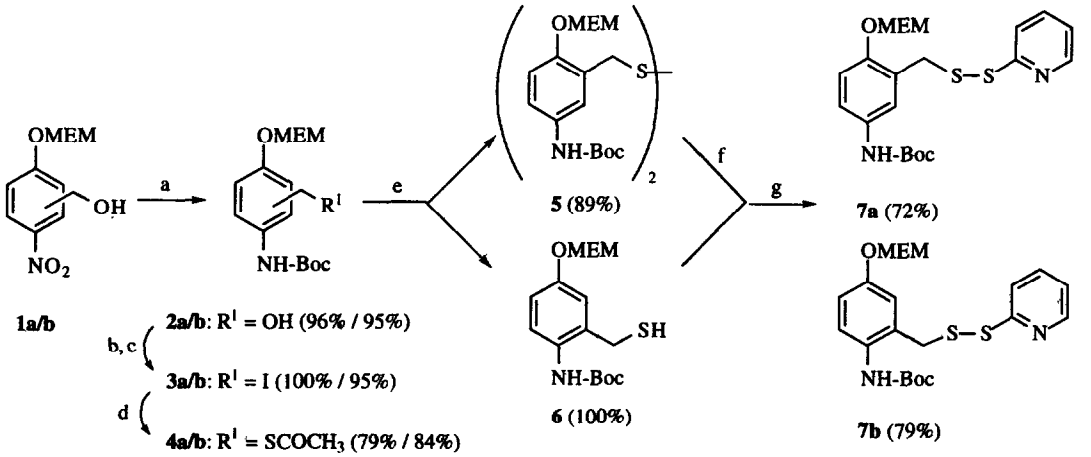
Abstract: New activated disulfides bearing a 4-diazocyclohexa-2,5-dienone precursor were synthesized in order to build up photoactivatable and cleavable peptides via cysteine modification.

Post-synthesis modification of peptides with arylazido or trifluoromethylaryldiazirine-derivatized bifunctional compounds is an usual but attractive route for rapidly obtaining peptidic photoprobes.[1] For this purpose, we designed new activated disulfides bearing a 4-diazocyclohexa-2,5-dienone (DCD) precursor for the derivatization of cysteine-containing peptides. In the dark, DCD are stable to various conditions excepted strong nucleophiles and can be conserved more than 24 hours at 25°C.[2] They strongly absorb at 350 nm ($\varepsilon > 25{,}000\ M^{-1}.cm^{-1}$). Upon irradiation they give highly reactive carbene species which insert in many groups including C-H bonds.[3] Moreover,they are consistent with an energy transfer activation process mediated by a protein-derived tryptophan residue which increases the selectivity of labelling.[4] We wish to report here the synthesis of pyridylsulfide (Pys) activated DCD precursors **7a** and **7b** suitable for building up photoactivatable peptides via cysteine modification.

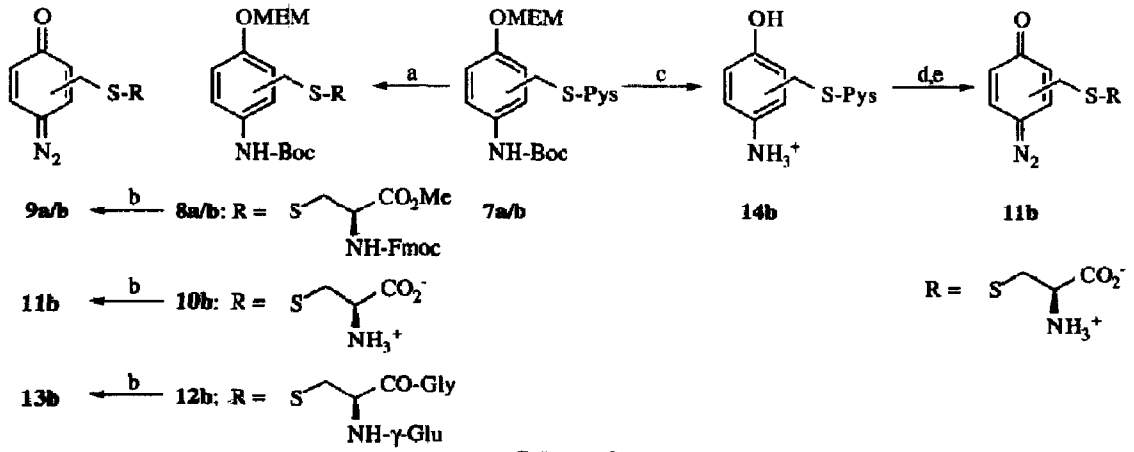


Scheme 1

a: H_2, Pd-C / MeOH; Boc_2O; b: CH_3SO_2Cl, NEt_3 / THF, 25°C; c: NaI, (4 equiv.) / acetone; d: CH_3COSH, NEt_3 / THF; e: MeONa (1 equiv.) / MeOH; f: PBu_3 (1.1 equiv.), H_2O (1 equiv.) / dioxane; g: Aldrithiol-2® / THF

The synthesis was achieved in 4 steps according to scheme 1: the previously described[2] nitrobenzylalcohols **1a** and **1b** were reduced by catalytic hydrogenation to the corresponding amines which were directly protected as Boc derivatives **2a/b**. Two steps iodination of the benzylalcohols and substitution of iodides **3a/b** by thioacetic acid in presence of triethylamine afforded the benzylthioacetates **4a/b**. Methanolysis of **4b** provided the stable mercaptan **6** which was directly activated with aldrithiol-2® as pyridyl disulfide derivative **7b**.[5] In contrast, similar treatment of the thioester **4a** led to the corresponding disulfide **5**: further activation required the preliminar reduction of the disulfide followed by one-pot activation.

Compounds **6** and **7a/b** can be stored several months at -18°C without detectable oxidation or disulfide exchange. No disulfide exchange was detected after 24 hours in organic solvent (THF, dioxane, DMF) or in neutral buffer. Oxidation of fluorenylmethyloxycarbonylcysteine methyl ester (Fmoc-Cys-OMe), cysteine and glutathione either with **7a/b**[6] or **14b**[7] was carried out in very good yields (figure 2, table 1). As a general rule, parahydroxyaniline derivatives are highly oxidizable species which must be handled under argon atmosphere: with the exception of **14b**, such compounds cannot be isolated and are diazotized *in situ*.



Scheme 2

a, b: see table 1; c: TFA-H_2O, 15 mn, 0°C under argon.

Acidic treatment, diazotization of disulfides and spectral characteristics of the corresponding DCD are summarized in table 1. Determination of their spectral characteristics showed they have similar properties to other DCD derivatives previously described.[2,8] Successful preparation of the modified glutathione **13b** unambiguously showed that diazotization of parahydroxyaniline has no effect on the terminal amine of the peptidic backbone provided one equivalent of reagent is employed. Compounds **9a/b**, **11b** and **13b** were conserved more than 24 hours at room temperature in a Tris buffer 0.1 M, pH 7.6 (C = 2 mM) or as the corresponding parahydroxyaryldiazonium (0.1 M in TFA) with less than 5% loss. In contrast, they readily decomposed when irradiated at 350 nm either in a buffer or in octanol which is commonly accepted as a mime of hydrophobic environment especially intramembranar area.

In order to introduce directly DCD derivatives in peptides, **14b** was diazotized (TFA, $NaNO_2$, 4°C; λ_{max} = 355 nm, ε = 35,000 $M^{-1}.cm^{-1}$). However DCD decomposed immediately in organic solvents and in neutral buffers in the dark when treated respectively with Fmoc-Cys-OMe or cysteine. In the same conditions, we observed that unsubstituted 4-diazocyclohexa-2,5-dienone reacts either with cysteine or pyridine-2-

mercaptan which is producted by Pys substitution: this result indicates that DCD compounds cannot be directly introduced in cysteine-containing peptides.

activated disulfides	thiols R-H [a]	yields (%)	disulfides	DCD [b]	λmax (nm)	ε ($M^{-1}.cm^{-1}$)	$t_{1/2}$ (350 nm)
7a	Fmoc-Cys-OMe [THF, overnight]	90	**8a**[9]	**9a**[10] [TFA-H_2O, 0.1 M $NaNO_2$]	355(2) 356(3)	29,000(2) 32,000(3)	27 s(2) 37 s(3)
7b	Fmoc-Cys-OMe [idem]	97	**8b**[9]	**9b**[10] [idem]	355(2) 357(3)	30,000(2) 32,000(3)	22 s(2) 36 s(3)
7b	Cys [DMF, 2 h]	100(1)	**10b**	**11b**[11] [idem]	355(2)	31,000(2)	12 s(2)
7b	Glutathione [DMF-Tris HCl 0.1M pH 7.6, 2 h]	95(1,4)	**12b**[12]	**13b**[13] [idem]	355(2)	30,500(2)	39 s(2)
14b	Cys [DMF, 2 h]	100(1)	non isolated	**11b** [Isoamyl nitrite / AcOH]	355(2)	32,000(2)	14 s(2)

Table 1

Synthesis and spectral characteristics of photoactivatable cysteine derivatives in Tris buffer and octanol; (*1*) Yields evaluated by HPLC; (*2*) in Tris HCl buffer 0.1M, pH 7.6; (*3*) In octanol; $E_{350\ nm} = 10^{-2}$ $mW.cm^{-2}$; (*4*) The other product is oxidized glutathione.

In conclusion, the use of protected Pys derivatives **7a**, **7b** and **14b** seems to be a valuable way for introducing DCD related photolabelling agents in peptides: in these compounds, the reactive position is located closer to the peptidic backbone than in most bifunctionnal photoactivatable agents; this should limitate the parasitic long-range labelling. Diazotization of p-hydroxyaniline has no effect on primary amines including lysine[2] and can be carried out on peptides in strong or mild acidic conditions.

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6. **7a**: ^1H NMR ($CDCl_3$) δ 8.40 (dd, J = 0.9 Hz, J' = 4.6 Hz, 1H), 7.54 (m, 2H), 7.19 (m, 2H) + 7.05 (d, J = 7.8 Hz, 1H) + 7.01 (dd, J = 1.5 Hz, J' = 11.7 Hz, 1H), 6.35 (broad s, 1H), 5.26 (s, 2H), 4.02 (s, 2H), 3.84 (m, 2H), 3.56 (m, 2H), 3.38 (s, 3H), 1.50 (s, 9H). MS (EI): m/z = 453 (M). Anal. calcd for $C_{21}H_{228}N_2O_5S_2$: C, 55.73 H, 6.24; N, 6.19. Found: C, 55.26; H, 6.21; N, 6.05.

7b: mp = 49-53°C. ^{1}H NMR ($CDCl_3$) δ 8.72 (d, J = 4.3 Hz, 1H), 7.96 (broad s, 1H), 7.61 (dd, J = 1.7 Hz, J' = 7.4 Hz, 1H) 7.55 (dd, J= 1.8, J = 8.4 Hz, 1H), 7.39 (dd, J = 0.9 Hz, J' =9.0 Hz, 1H), 7.12 (ddd, J = 0.9 Hz, J' = 1.7 Hz, J" = 7.4 Hz, 1H), 6.96 (m, 2H), 5.21 (s, 2H), 4.11 (s, 2H), 3.79 (m, 2H), 3.55 (m, 2H), 3.38 (s, 3H), 1.53 (s, 9H). MS (EI): m/z = 453 (M). Anal. calcd for $C_{21}H_{228}N_2O_5S_2$: C, 55.73 H, 6.24; N, 6.19. Found: C, 55.43; H, 6.33; N, 6.17.

7. **14b:** 8.41 (m, 1H), 8.11 (broad t, J = 8.0 Hz, 1H), 7.78 (d, J = 8.2, 1H), 7.57 (broad t, J = 6.5 Hz, 1H), 7.12 (d, J = 8.6 Hz, 1H), 6.76 (broad s, 1H), 6.61 (dd, J = 2.7 Hz, J' = 8.6 Hz, 1H).
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9. **8a**: $[\alpha]_D^{25}$ = +8° (C = 1, chloroform). Anal. calcd for $C_{35}H_{42}N_2O_9S_2$: C, 60.15 H, 6.06; N, 4.01. Found: C, 59.96; H, 6.31; N, 4.06.
   8b: $[\alpha]_D^{25}$ = +19° (C= 1, chloroform). Anal. calcd for $C_{35}H_{42}N_2O_9S_2$: C, 60.15 H, 6.06; N, 4.01. Found: C, 60.22; H, 5.95; N, 3.88.
10. **9a**: ^{1}H NMR ($CDCl_3$) δ 8.37 (dd, J = 2.6 Hz, J' = 8.9 Hz, 1H), 7.87 (m, 2H), 7.72 (broad d, 2H), 7.40 (m, 5H), 7.02 (d, J = 8.9 Hz, 1H), 4.60 (m, 1H), 4.39 (m, 2H), 4.26 (m, 3H), 3.73 (s, 3H), 3.10 (m, 2H).
    9b: ^{1}H NMR ($CDCl_3$) δ 8.52 (d, J = 9.3 Hz, 1H), 7.87 (d, J = 7.3 Hz, 2H), 7.72 (d, J = 7.3 Hz, 2H), 7.43 (m, 3H), 7.25 (dd, J = 2.5 Hz, J' = 9.3 Hz, 1H), 4.58 (m, 1H), 4.42 (s, 2H), 4.25 (m, 3H), 3.73 (s, 3H), 3.14 (dd, J = 5.2 Hz, J' = 13.9 Hz, 1H), 2.98 (dd, J = 8.8 Hz, J' = 13.9 Hz, 1H).
11. **11b**: Corresponding diazonium ^{1}H NMR (D_2O) δ 8.26 (d, J = 9.3 Hz, 1H), 7.07 (d, J = 2.2 Hz, 1H), 6.99 (dd, J = 2.2 Hz, J' = 9.3 Hz, 1H), 4.00 (dd, J = 4.7 Hz, J' = 6.8 Hz, 1H), 2.94 (m, 2H).
12. **12b:** HPLC (analytical column Nucleosil C_{18} 10 µm, linear gradient 0.1% TFA-acetonitrile 100-0 to 0-100 in 15 min): rt = 10.9 min at 230 nm. ^{1}H NMR (D_2O) δ 7.27 (d, J = 8.6 Hz, 1H), 7.12 (d, J = 2.6 Hz, 1H), 7.04 (dd, J = 2.6 Hz, J' = 8.6 Hz, 1H), 5.32 (s, 2H), 4.52 (t, J = 7.1 Hz, 1H), 4.01 (m, 1H), 3.95 (s, 2H), 3.94 (m, 2H), 3.84 (m, 2H), 3.59 (m, 2H), 3.30 (s, 3H), 2.53 (m, 4H), 2.20 (m, 2H), 1.47 (s, 9H); MS (ES): m/z = 649.1 (MH^+).
13. **13b:** (associated diazonium) HPLC (same conditions): rt = 8.2 min at 230 and 315 nm; λ_{max} = 316 nm; ^{1}H NMR (D_2O) δ 8.35 (d, J = 9.2 Hz, 1H), 7.17 (d, J = 1.7 Hz, 1H), 7.12 (dd, J = 1.7 Hz, J' = 9.2 Hz, 1H), 4.80 (dd, J = 4.0 Hz, J' = 9.6 Hz, 1H), 4.05 (t, J = 6.5 Hz, 1H), 3.99 (s, 2H), 3.97 (s, 2H), 3.40 (dd, J = 4.0 Hz, J' = 14.5 Hz, 1H), 3.26 (dd, J = 9.5 Hz, J' = 14.5 Hz, 1H), 2.54 (m, 2H), 2.16 (m, 2H).

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