Bioorganic & Medicinal Chemistry Letters, Vol.1, No.10, pp. 519-522, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

DESIGN AND SYNTHESIS OF A POTENTIAL AFFINITY/CLEAVING REAGENT FOR BETA-PLEATED SHEET PROTEIN STRUCTURES

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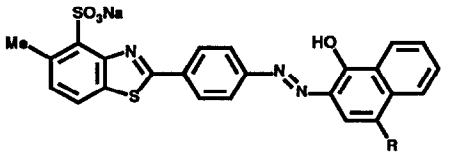
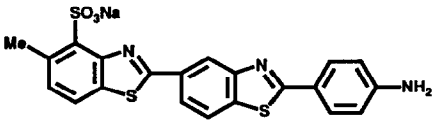
(*Received* 1 *August* 1991)

Abstract: The design and synthesis of a bifunctional reagent, 1, is described. This compound binds to beta-pleated sheet protein structures, while providing a complexation site for ferrous ion. These immobilized ions may be used to generate reactive, diffusible chemical species at or near the protein surface.

The design of small molecules which bind to, and subsequently cleave biomacromolecules with enzyme-like specificity has been elegantly demonstrated by Dervan et al.[1] with a series of reagents which may be considered mimics of the endonucleases. The principle has been extended to the recognition and cleavage of proteins, using modified ligands with high affinity for specific binding sites on the target structures.[2] We became interested in the degradation of structural proteins, which may lack specific binding sites for high affinity ligands, but can be distinguished from other proteins by their secondary structure. These include the proteins which comprise the plaques and tangles of Alzheimer's disease brain which, like silk proteins, adopt a characteristic beta-pleated sheet conformation.[3] In this paper, we describe the design and synthesis of a reagent, 1, which recognizes the beta-pleated sheet motif in certain structural proteins, and report preliminary results on the utilization of 1 in the degradation of these structures.



1 R = $(CH_2)_3NHCO$ with CO_2Na, NaO_2C, CO_2Na



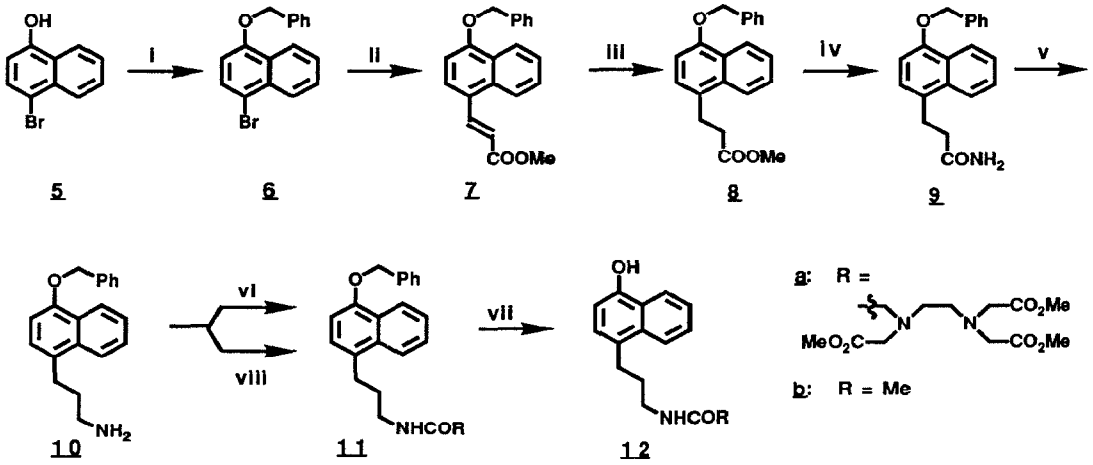
4 (Primulin)

2 R = $(CH_2)_3NHCOCH_3$

3 R = SO_3Na (Thiazin Red)

Our work began with the observation that the fluorescent dyes thiazin red, 3, and primulin, 4, bound reversibly but with high affinity to tangles purified from Alzheimer's brain tissue.[4] We sought a method to solubilize peptides from these tangles, employing a bifunctional molecule possessing a protein binding domain linked to an Fe^{+2} ion chelator which could generate reactive, diffusible chemical species capable of protein degradation.[5] By evaluating close analogs of structures 3 and 4, we determined that naphthol-based azo structures offered the promise of higher affinity for target proteins as well as greater latitude in synthesis of bifunctional molecules. We further determined that sulfonation of the benzothiazole was

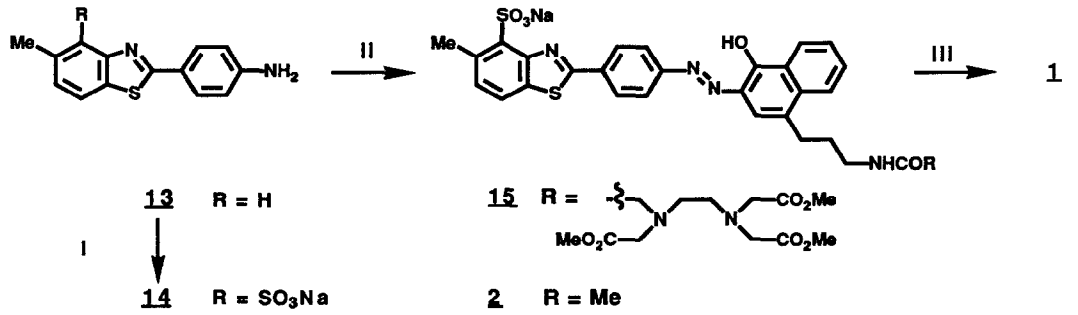
essential for binding activity (as well as good aqueous solubility), but that sulfonation in the naphthol ring of **3** was unnecessary. We therefore proposed that the 4-position of the naphthol moiety could be substituted without interfering with binding properties of the parent azo dye. Structure **1** was therefore chosen as our synthetic target, along with the N-acetyl compound **2** which could be employed in control experiments. We planned a convergent synthesis of **1**, employing a conventional azo dye coupling[6] between an appropriately functionalized naphthol **12a** and the diazonium ion derived from the sulfonated benzothiazole **14**. Naphthol **12a** and its N-acetyl analog **12b** were prepared in a seven step sequence from the known[7] 4-bromo-1-naphthol according to Scheme 1. As a key step in this synthesis, we found that Weinreb amidation[8] employing the tetramethyl ester of EDTA[9] furnished the monoamide in acceptable yields, obviating the need for the triester monoacid which had been employed by previous workers.[1,2]



Scheme 1. (i) $PhCH_2Br$, K_2CO_3, DMF (90%); (ii) methyl acrylate, Et_3N, $P(o\text{-}tol)_3$, $Pd(OAc)_2$ (66%); (iii) Mg, MeOH (83%); (iv) NH_4Cl, $AlMe_3$, $PhCH_3$, (94%); (v) $LiAlH_4$, THF (90%); (vi) Me_3Al, then Me_4EDTA, $PhCH_3$ (38%); (vii) NH_4HCO_2, Pd/C, MeOH (77-84%); (viii) Ac_2O, pyridine (89%).

Sulfonation of 2-(4-aminophenyl)-6-methylbenzothiazole **13** with fuming sulfuric acid afforded the desired 7-position sulfonic acid product **14**, along with traces of the 5-sulfonic acid isomer. Diazotization and coupling with naphthols **12a** or **b** afforded the deep red azo dye products **15** and **2**, respectively. Saponification of **15**, followed by purification by reverse-phase chromatography, provided the desired bifunctional reagent **1** (Scheme 2).[10]

The affinity of compounds **1**, **2**, and **15** for beta-pleated sheet structures was conveniently demonstrated by incubation with silk from *Bombyx mori* coccoons.[11] Silk fibers rapidly bound these red azo dyes, as well as compound **4**, with high affinity. The fluorescent properties of **1**-**4** made it possible to observe similar effects in fluorescence microscopy of purified tangles from Alzheimer's brain.[12] Azo dyes **1** and **2**, as well as primulin, **4**, exhibited half-maximal binding to tangle proteins at concentrations of



Scheme 2. (I) fuming H_2SO_4 (34%); (II) $NaNO_2$, H_2O, HOAc, HCl, then 12a or b (47-50%); (III) NaOH, MeOH/H_2O, then purification according to ref. 10 (90%).

about 100 nM, and were only slightly less potent than thiazin red, 3. This binding was saturable and reversible, as evidenced by the ability of compounds 1-4 to displace each other in a concentration-dependent manner.[13]

We have used silk protein in a model study to determine the utility of 1 as a protein cleavage reagent in the presence of Fe^{+2} and reducing agents. Once bound to silk protein, 1 readily absorbed Fe^{+2} ion from aqueous solutions. Silk treated with 2 absorbed a much lower, though significant quantity of Fe^{+2}, perhaps via complexation with the naphthol/azo linkage. The complex of 1, Fe^{+2}, and silk protein was apparently stable until the addition of dithiothreitol, which caused a concentration-dependent darkening of the sample within 1-2 hours. This was followed by bleaching of the red dye chromophore over a more extended period. This latter phenomenon was not observed if either the Fe^{+2} or dithiothreitol was omitted, or if 2 was substituted for 1. This destruction of the bound reagent was taken as evidence that reactive species such as hydroxyl radical[5] had been generated by 1 under these conditions. However, microscopic examination of the silk fibers revealed no gross morphological differences except an apparent reduction in rigidity as compared to controls.[14]

In conclusion, we have prepared 1 as an analog of thiazin red which retains affinity for beta-pleated sheet proteins, while providing a complexation site for transition metal ions such as Fe^{+2}. We believe that further studies are warranted on the utility of 1 as a reagent for protein cleavage.

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10 Satisfactory spectral data were obtained for all new compounds. Structure and purity determinations were based on ^{1}H NMR, reverse phase TLC, reverse phase HPLC, IR, UV, and FAB mass spectral data. Final product 1 was freed of adventitious transition metal contaminants by treatment with a large excess of disodium EDTA at pH 2, neutralization, and purification by reverse phase column chromatography on C_{18}-modified silica gel (Bodman) in aqueous methanol.
For 1, ^{1}H NMR (DMSO-d_6, 400 MHz): (free acid obtained upon 2N HCl treatment of Na salt)
1.86, m, J=7.6 Hz, 2H; 2.70, s, 3H; 2.87, t, J=7.7 Hz, 2H; 3.21, t, J=4.7 Hz, 2H; 3.26, m, 2H; 3.34, t, J=5.0 Hz, 2H, 3.76, s, 4H; 4.00, s, 2H; 4.11, s, 2H; 7.19, s, 1H, 7.36, d, J=8.1 Hz, 1H; 7.59, t, J=7.6 Hz, 1H; 7.79, t, J=7.6 Hz, 1H; 7.86-7.90, m, 4H; 8.20, d, J=8.6 Hz, 2H; 8.38, d, J=7.7 Hz, 1H; 8.70, t, NH. UV: λ_{max} = 346, 520 nm. IR: (KBr, cm^{-1}) 3420, s; 1640, s; 1605, s; 1400, s.
For 2, ^{1}H NMR (DMSO-d_6, 300 MHz): 1.79, m, 2H; 1.85, s, 3H; 2.70, s, 3H; 2.83, m, 2H; 3.18, m, 2H; 7.16, s, 1H; 7.36, d, J=8.2 Hz, 1H; 7.58, t, 1H; 7.78, t, 1H; 7.84-7.90, m, 4H; 8.00, t, NH; 8.19, d, J=8.4 Hz, 2H; 8.38, d, J=7.8 Hz, 1H. UV: λ_{max} = 346, 512 nm. FAB: m/e = 597 (M+1); 619 (M+1+Na). IR: (KBr, cm^{-1}) 3420, s; 1640, m; 1605, m; 1200, s.

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13. Fluorescence microscopy with compounds 1-3 employed excitation in the range of 530-560 nm, monitoring red light emission above 580 nm. For 4, excitation was in the range 270-380 nm, with observation of yellow fluorescence between 410 and 580 nm. The emission spectrum of 4 in aqueous solution showed a maximum at 515 nm; this shifted to 470 nm on binding to Alzheimer's tangles

14. Silk samples (ca. 1 cm^2) were incubated with 1 or 2 (1 mg/mL, 1:1 MeOH/H2O) for 5 min, and were then rinsed free of excess reagent. These were then treated with freshly prepared $FeSO_4 \cdot 7H_2O$ solution (3.6 mM) for 5 min, and again rinsed with water. Samples could be rinsed with Na_2EDTA solutions at this point without removing Fe^{+2} bound to adsorbed 1. Samples were then incubated with dithiothreitol (1-30 mM) in 50 mM NaCl/10 mM Tris HCl buffer, pH 8-8.5.