calmodulin is incubated with equivalent concentrations of DTT, Fe(II) and DTT, or Fe(II)-EDTA and DTT (lanes 2-4). Very little cleavage is generated by TFE-Fe in the absence of DTT (lane 8), and none is generated by TFE-Mg(II) in the presence of DTT (lane 9).[15] Cleavage by TFE is dependent on the presence of oxygen. If oxygen is removed from the reaction mixture with an argon purge, far less cleavage is observed (lane 10). Addition of catalase (100 μg/mL) to the reaction mixture eliminated protein cleavage, while boiled catalase had no effect (data not shown).[16] These results are consistent with oxidative cleavage of the protein backbone by TFE-Fe.[17]

Several experiments were performed to determine whether cleavage was occurring in the trifluoperazine binding pocket. At saturation, calmodulin stimulates by 3-10-fold the rate of phosphodiesterase-catalyzed hydrolysis of cyclic AMP.[18] A concentration of 75 μM TFE or TFE-Mg^{2+} was required to reduce this stimulation by 50% (IC_{50} = 75 μM).[19] TFP shows a corresponding IC_{50} of 15 μM. Although TFE is 5 times less effective than TFP, an IC_{50} of 75 μM is well within the range observed for phenothiazine ligands.[10] Therefore, TFE and TFE-Mg^{2+} are calmodulin antagonists.

A rigorous test for any affinity reagent is a competition experiment. Site-specific binding/cleaving demands that cleavage be attenuated by the original ligand. To test whether TFE would fulfill this requirement, calmodulin was incubated with 400 μM trifluoperazine, 40 μM TFE-Fe, and 30 mM DTT.[20] Comparison of lanes 7 and 11 in Figure 3 shows clearly the absence of cleavage in lane 11.

A mandatory test for affinity cleavage of calmodulin recognizes that the structure and activity of this protein are tied intimately to the concentration of Ca^{2+} in the medium. At saturation, calmodulin binds four Ca^{2+} ions in two EF-hand domains.[21] Only in this Ca^{2+}-bound form does calmodulin activate cellular enzymes or bind trifluoperazine drugs.[22] Therefore, TFE-Fe should be unable to cleave calmodulin in the absence of Ca^{2+}. Indeed, no protein cleavage is observed when Ca^{2+} is omitted from the reaction mixture (lane 12). Taken together, these experiments demonstrate that TFE-Fe is an affinity cleavage reagent for calmodulin. Studies directed toward determination of the precise cleavage sites, analysis of other cleaving reagents, and the evaluation of the power of these reagents as potent *receptor modifying/damaging reagents* are in progress.

Acknowledgment. A.S. gratefully acknowledges the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this work. We thank Dr. William Hait and Joan Gesmonde for assistance with the calmodulin assays.

Supplementary Material Available: Spectroscopic characterization of compounds **1-4** and combustion analysis of **1** and **4** (1 page). Ordering information is given on any current masthead page.

(15) Fe(II) does induce nonspecific protein cleavage in the absence of TFE; however, millimolar concentrations are required. Discrete bands are not observed under these conditions; the products appear as a diffuse smear on the gel.

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(19) The IC_{50} for TFE-Fe(II) could not be determined due to competitive inhibition of phosphodiesterase. Neither TFP, TFE, nor TFE-Mg^{2+} (100 μM) inhibited phosphodiesterase in the absence of calmodulin.

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A New Strategy for Selective Protein Cleavage

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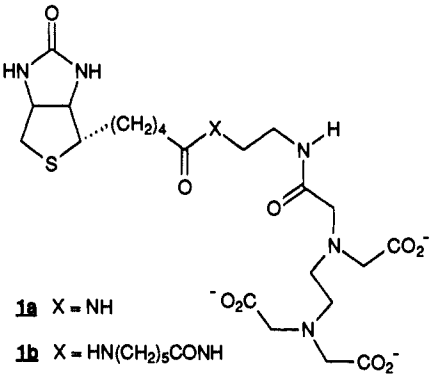
The ability of proteolytic enzymes and chemical reagents to selectively cleave peptides and proteins at defined sequences has greatly facilitated studies of protein structure and function.[1] Unfortunately, only a limited number of selective peptide cleavage agents exist, in contrast to the wide array of selective nucleases available for analyzing and manipulating nucleic acid structure. The development of strategies for generating site-specific peptidases of any defined sequence would greatly facilitate the mapping of protein structural domains, protein sequencing, the generation of semisynthetic proteins, and would likely lead to the development of new therapeutic agents. We report here a new approach to the generation of selective protein cleavage agents that is based on oxidative cleavage of the polypeptide backbone.[2] Attachment of the metal chelator ethylenediaminetetraacetic acid (EDTA) to biotin allows delivery of redox-active Cu^{2+} or Fe^{3+} to the binding site of streptavidin and selective cleavage of the polypeptide backbone. Similarly, attachment of redox-active chelates to other ligands or to antibody combining sites may lead to a new class of affinity cleavage agents,[3] to the design of catalytic drugs, or to the generation of a family of sequence-selective peptidases.

Chemical studies have demonstrated that cupric ion can catalyze the cleavage of peptide and proteins by oxidation of the polypeptide backbone.[4] In addition, the metal-dependent enzyme peptidylglycine monooxygenase catalyzes the oxidative cleavage of glycine from the C-terminus of peptides.[5] Consequently, we reasoned that attachment of a Cu^{2+} chelate to biotin should result in selective cleavage of streptavidin in close proximity to the biotin binding site (just as EDTA·Fe^{2+} conjugates of DNA binding ligands oxidatively cleave nucleic acids[6]). The 72-kDa tetrameric protein streptavidin binds one biotin per monomer with an association constant of 10^{15} M^{-1}.[7] Biotin-EDTA derivatives **1a** and **1b** were prepared by treatment of (+)-biotin *N*-hydroxysuccin-



1a X = NH

1b X = $HN(CH_2)_5CONH$

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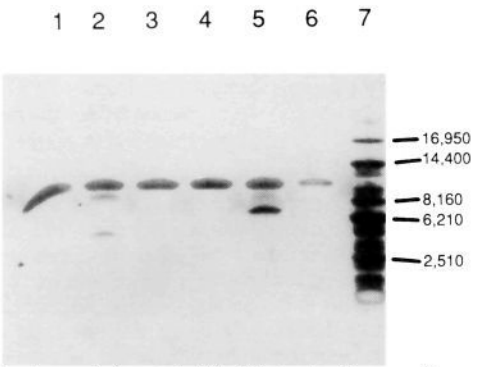

Figure 1. Lanes 1-6 contain 20 μM streptavidin core (in monomer). Lane 2: 20 μM $CuCl_2$. Lane 3: 20 μM $CuCl_2$, 20 μM EDTA. Lane 4: 20 μM $ZnCl_2$, 20 μM **1a**. Lane 5: 20 μM $CuCl_2$, 20 μM **1a**. Lane 6: 1 mM (+)-biotin, followed by 20 μM $CuCl_2$, 20 μM **1a**. Lane 7: horse heart myoglobin fragments. Cleavage experiments were carried out under aerobic conditions by complexation of $FeCl_3$ or $CuCl_2$ to **1a** followed by addition of the complex to streptavidin in 100 μL (final) 50 mM borate, pH 7.5. After 10 min, 2-mercaptoethanol was added (10% w/v), and the reactions were heated at 90 °C for 5 min and analyzed by gel electrophoresis.

imide ester with the appropriate triethyl ester EDTA derivative in CH_3CN,[8] followed by hydrolysis with LiOH and chromatography on DEAE-Sephadex with a 0.01-1.5 M NH_4HCO_3 gradient.[9,10]

Cleavage experiments were performed by complexation of Cu^{2+} (or Fe^{3+}) with an equimolar amount of **1a** or **1b** followed by addition to 1 molar equiv of streptavidin core in the presence of oxygen (streptavidin was from Sigma and is a proteolyzed core streptavidin of approximately 14 kDa per monomer that is missing 13 N-terminal and 20 C-terminal amino acids[11]). Mercaptoethanol was then added, the sample was heated to 90 °C for 5 min, and the reaction products were analyzed by gel electrophoresis[12] with silver staining.[13] Examination of the cleavage gel (Figure 1) reveals that the biotin-EDTA·Cu^{2+} complex (**1a**·Cu^{2+}) selectively cleaves streptavidin core to produce a 7-kDa fragment (the **1a**·Fe^{3+} complex generates the same cleavage band). The streptavidin:7-kDa-fragment ratio was 3:1 by quantitation of the silver-stained bands.[14] The lack of additional bands (corresponding to other cleavage fragments) may be due to comigration of two 7-kDa cleavage fragments or to secondary cleavage reactions. In contrast to cleavage by the **1a**·Cu^{2+} complex, incubation of streptavidin with Cu^{2+}·EDTA, Fe^{3+}·EDTA, or the preformed complex of **1a** with the non-redox-active metal Zn^{2+} does not afford any cleavage. In addition, cleavage of streptavidin by the **1a**·Cu^{2+} complex is completely inhibited by addition of 1 mM (+)-biotin. Cleavage of streptavidin by uncomplexed Cu^{2+} affords lower yields of two fragments that are different from those generated by **1a**·Cu^{2+} (probably due to chelation of Cu^{2+} by protein side chains or backbone[4]). Uncomplexed Fe^{3+} produces no cleavage. The complex of either Cu^{2+} or Fe^{3+} with **1b**, which has a seven-chain tether, does not cleave protein.

These results are consistent with the notion that the biotin-EDTA conjugate **1a** selectively delivers redox-active Cu^{2+} or Fe^{3+} in close proximity to the polypeptide backbone at the biotin binding site of streptavidin, resulting in selective protein cleavage at that site. Examination of the three-dimensional structure of streptavidin suggests that 7-kDa fragment(s) would result from cleavage within the sequence Trp 79-Ala 89 at the biotin binding site.[7] Finally, pyruvic acid and alanylamide are generated by cleavage of the dipeptide Ala-Ala with EDTA·Fe^{3+} in the presence of O_2 and mercaptoethanol. These products are consistent with an oxidative cleavage mechanism involving initial oxidation of the α carbon to the α-hydroxylated product, with subsequent cleavage.[4,15] Additional experiments into the nature and stoichiometry of the cleavage reaction are in progress.

Acknowledgment. We acknowledge support of this work by the Department of Energy (HA020203) and The Lucille P. Markey Trust and thank W. Hendrickson for helpful discussions.

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Synthesis of Two Noninterconvertible Conformers of a Single Host. Self-Filled and Vaulted Cappedophanes

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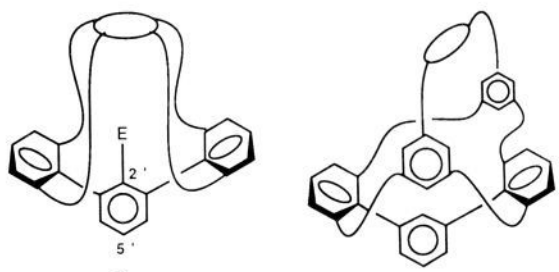
We recently described the synthesis of *m*-terphenyl-based cyclophanes **1**, for which we coined the name *cappedophanes*.[1] Our one-pot route to the *m*-terphenyl moiety of **1** permits the direct introduction of substituents E at C2′.[2] In our first studies, however, the tethers to the aromatic cap were too short (only two or three atoms each) to permit E to be larger than a proton.[3]



1 2

The aim of the present work was to construct a vaulted cappedophane of sufficient volume to accommodate a larger E, so that functional group chemistry inside and outside a cavity with a controlled microenvironment could be compared. To do this, it was essential to provide rigid cavity walls, because simply increasing the number of atoms in the tethers of **1** would make the cap collapsible and reduce the enclosed volume.[4] Consequently, we set molecules such as **2**, with aromatic cavity walls and cap, as our target.

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