A cofactor approach to copper-dependent catalytic antibodies

Kenneth M. Nicholas*†, Paul Wentworth, Jr.‡, Curtis W. Harwig‡, Anita D. Wentworth‡, Asher Shafton‡, and Kim D. Janda†‡

*Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK 73019; and ‡Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

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A strategy for the preparation of semisynthetic copper(II)-based catalytic metalloproteins is described in which a metal-binding bis-imidazole cofactor is incorporated into the combining site of the aldolase antibody 38C2. Antibody 38C2 features a large hydrophobic-combining site pocket with a highly nucleophilic lysine residue, Lys^{H93}, that can be covalently modified. A comparison of several lactone and anhydride reagents shows that the latter are the most effective and general derivatizing agents for the 38C2 Lys residue. A bis-imidazole anhydride (5) was efficiently prepared from *N*-methyl imidazole. The 38C2–5-Cu conjugate was prepared by either (*i*) initial derivatization of 38C2 with 5 followed by metallation with $CuCl_2$, or (*ii*) precoordination of 5 with $CuCl_2$ followed by conjugation with 38C2. The resulting 38C2–5-Cu conjugate was an active catalyst for the hydrolysis of the coordinating picolinate ester 11, following Michaelis–Menten kinetics [$k_{cat}(11) = 2.3\ min^{-1}$ and $K_m(11)$ 2.2 mM] with a rate enhancement [$k_{cat}(11)k_{uncat}(11)$] of 2.1×10^5. Comparison of the second-order rate constants of the modified 38C2 and the Cu(II)-bis-imidazolyl complex $k(6\text{-}CuCl_2)$ gives a rate enhancement of 3.5×10^4 in favor of the antibody complex with an effective molarity of 76.7 M, revealing a significant catalytic benefit to the binding of the bis-imidazolyl ligand into 38C2.

Catalysis at metal centers plays a key role in both enzymatic and abiological reactions, providing reaction pathways, rates, and selectivity often unattainable from conventional acid, base, or nucleophilic catalysis. However, traditionally separate disciplines, the study of natural (i.e., enzymatic) and synthetic (nonbiological) catalysts has interfaced with the genesis of semisynthetic and *de novo* proteins (1). A number of methods have been explored for producing such hybrid species that possess metal centers, including: (*i*) attachment of a synthetic metal-containing cofactor to a protein (2–4); (*ii*) antibody elicitation using a metal-binding hapten/protein conjugate (5–10); (*iii*) site-directed mutagenesis of proteins and antibodies to incorporate metal-binding sites (11–15); and (*iv*) panning of antibody libraries for metal binding using immobilized metal complexes (16, 17).

Our approach is directed toward the design and generation of novel metallo-catalytic antibodies and is inspired by the active-site structures of the many metalloenzymes that possess a coordination sphere with two or more histidine-derived imidazole ligands. The most important catalytic functions of such polyhistidyl enzymes are hydrolytic or oxidative. Illustrative of the numerous and structurally diverse polyhistidyl Zn-hydrolases (18) is carboxypeptidase A, which catalyzes the hydrolysis of C-terminal amino acid residues and features a bis-histidine/glutamate (CO_2^-) coordination sphere and the nucleophilic assistance of a nearby Glu (19). The Cu-hydroxylases, dopamine β-hydroxylase and peptide amidating hydroxylase (20), are chemically extraordinary in effecting the regio- and enantioselective hydroxylation of weakly activated C—H bonds, a transformation with little precedent among nonenzymatic copper catalysts (21, 22). The active sites of hydroxylase enzymes possess two Cu-polyhistidine centers, one of which appears to serve as an electron or superoxide transfer site and the other at which the substrate is hydroxylated (23–25). The physiological importance of these enzymes continues to evoke intense efforts to elucidate their molecular mechanisms, to mimic their structure and function, and to discover mechanism-based inhibitors for biomedical applications (26–27).

Numerous structural and functional model compounds for the active site of Zn-hydrolase enzymes have been prepared (18), but few possess the imidazole (histidine) or carboxylate ligands of the enzymes. More sophisticated model systems have incorporated hydroxyl- and carboxyl-containing tethers to serve as nucleophilic or protic functions (28, 29), micelles (30), and cyclodextrins (31, 32) to simulate the protein environment of metalloenzymes. Substantial rate accelerations for hydrolysis, particularly of carboxylic acid esters, have been attained with some of these model Cu(II) and Zn(II) complexes (33–36). Although Cu complexes, which reasonably mimic the structural and spectroscopic features of the Cu-hydroxylases, have been prepared (37–40), few possess polyimidazole ligands or have been shown to promote the hydroxylation of relevant substrates (usually stoichiometric hydroxylation of their own ligands is a major problem) (41–44), and none of these reactions are catalytic.

Our initial efforts in this area have focused on the preparation of a metal-binding bis-imidazolyl ligand and the corresponding metal complex cofactor that features high-affinity metal–ligand binding ($K_d \approx 10^{-12}$) (45). The metal cofactor is then incorporated into the combining site of the well-characterized monoclonal catalytic antibody 38C2 (46) to allow investigation of the catalytic potential of the biomimetic bis-imidazole coordination cofactor. We reasoned that the resulting semisynthetic metalloantibodies would ideally possess the catalytic function of the metal center enhanced by the unique microenvironment characteristics imparted by the antibody combining site.

Antibody 38C2 was selected as the Ig for modification because it possesses a large promiscuous hydrophobic combining a site pocket that contains a highly nucleophilic low-pKa lysine residue, Lys^{H93}, which can be covalently modified (47, 48).

Materials and Methods

Monoclonal antibody 38C2 was obtained from The Scripps Research Institute. The antibody was routinely purified in a three-stage process that included precipitation into ammonium-sulfate protein-G affinity column chromatography and ion-exchange column chromatography. The resultant protein

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

†To whom reprint requests may be addressed. E-mail: kmnicholas@chemdept.chem.ou.edu or kdjanda@scripps.edu.

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preparation was >99% pure as determined by SDS/PAGE analysis and silver staining. Protein concentration was routinely measured by UV spectroscopy {1.35 × (OD at 280 nm) = [protein] mg/ml}. Conversion to molar concentration assumed a molecular weight of a whole IgG = 150,000 and two binding sites per antibody molecule. All buffers and salts were obtained from commercial sources. Doubly distilled nanopure water was used throughout. Dialysis was conducted by using microdialysis cassettes (10,000 molecular weight block) (Pierce). UV-Vis, NMR (300 MHz), IR, and mass spectra (electrospray ionization) were performed on standard instruments. Inductively coupled-plasma-MS analysis (for Cu) was carried out by Elemental Research (Vancouver). Lactones **2a–2e** and anhydrides **3a–3c** were obtained from Aldrich and used without further purification. Anhydride **3d** (49), **3e** (50), (*S*)-(+)-decalenone derivative **4a** (51), 4-nitrophenyl-2-picolinate 11 (33), and bis-imidazole carbinol **6** (45) were prepared according to reported procedures. The titration of Lys^{H93} enone formation by reaction of 38C2 with pentane-2,4-dione at 318 nm was performed as previously described (46, 47).

Preparation of 2-(*S*)-(4-Nitrophenoxycarbonylamino)succinic Acid Di-*tert*-butyl Ester (7). Di-*tert*-butyl aspartate hydrochloride (0.80 g, 2.8 mmol) and *p*-nitrophenyl chloroformate (0.68 g, 3.4 mmol) were dissolved in dry CH_2Cl_2 (25 ml) and stirred under an inert atmosphere (argon). *N*-ethyl morpholine (0.72 ml, 5.7 mmol) was added by syringe. After stirring for 4 h, the solution was washed twice with equal volumes of cold aqueous $NaHCO_3$ and then cold brine. The organic phase was dried over $MgSO_4$, rotary evaporated, and then dried *in vacuo* to provide the carbamate **7** (1.30 g). NMR analysis routinely indicated that **7** was ≈90% pure with a small amount of *p*-nitrophenol present. A pure sample of **7** was obtained by silica gel chromatography, eluting with ethyl acetate; ^{1}H NMR ($CDCl_3$) δ 8.2 (d, 8 Hz, 2H), 7.3 (d, 8 Hz, 2H), 6.2 (d, J = 6 Hz, 1H), 4.5 (m, 1H), 3.0 (dd, J = 12, 4 Hz, 1H), 2.8 (dd, J = 12, 4 Hz, 1H), 1.55 (s, 9H), 1.50 (s, 9H): ^{13}C NMR ($CDCl_3$) δ 159.8, 157.5, 145.2, 124.3, 124.0, 122.7, 121.0, 59.3, 48.6, 35.9, 24.3, 24.1; HRFABMS $C_{19}H_{27}N_2O_8$ $(M+H)^+$ calcd. 411.1768; obsd. 411.1770.

Preparation of Di-*tert*-butyl-*N*-aspartyl-bis-2-imidazolyl Carbamate (8). Sodium hydride dispersion (60% in mineral oil, 0.050 g, 1.25 mmol) was washed three times with hexane (2 ml) under argon, and then anhydrous tetrahydrofuran (THF) (10 ml) was added. To this stirred mixture was added bis-*2*-imidazolyl carbinol **6** (0.21 g, 1.1 mmol) (note: gas evolution accompanies the evolution). After 30 min, a solution of **7** (0.65 g, 1.3 mmol) in THF (7 ml) was added, and the mixture was allowed to stir overnight. The orange reaction mixture was then diluted with an equal volume of ethyl acetate, and the mixture was washed several times with aqueous $NaHCO_3$. The organic phase was dried over $MgSO_4$ and concentrated to dryness *in vacuo*. The crude residue was purified by silica gel chromatography [EtOAc (1% Et_3N), followed by 1:6 MeOH/EtOAc (1% Et_3N)] to afford product **8** (0.35 g, 68% yield based on **6**) as a white solid. ^{1}H NMR ($CDCl_3$, δ) 7.2 (s, 1H), 7.0 (d, 2 Hz, 2H), 6.8 (d, J = 2 Hz, 2H), 6.0 (d, J = 6 Hz, 1H), 4.4 (m, 1H), 3.85 (s, 3H), 3.8 (s, 3H), 2.9 (dd, J = 12, 4 Hz, 1H), 2.7 (dd, J = 12, 4 Hz, 1H), 1.45 (s, 18H)]; ^{13}C NMR ($CDCl_3$, δ) 170.8, 170.1, 155.1, 143.4, 128.3, 123.3, 66.2, 51.6, 38.2, 34.3, 28.6; MS (electrospray ionization, MeOH) m/e 464 ($M+H^+$ for $C_{22}H_{32}N_5O_6$).

Preparation of Diacid 9. To a stirred solution of ester **8** (0.21 g, 0.45 mmol) in dry CH_2Cl_2 (3 ml), trifluoroacetic acid (2 ml) was added. After 5 h at room temperature, HPLC analysis (C_{18}, 1:4 acetonitrile/water, 1% trifluoroacetic acid) indicated that the reaction was complete. The volatiles were removed by rotary evaporation, and the residue was dried under vacuum to give the diacid **5** (di-trifluoroacetate salt) as an oily colorless solid (0.26 g, 100%). ^{1}H NMR (CD_3CN, δ) 7.3 (bs, 4H), 7.2 (s, 1H), 6.8 (d, J = 6 Hz, 1H), 4.4 (m, 1H), 3.8 (s, 6H), 2.8 (m, 2H); ^{13}C NMR ($CDCl_3$, δ) 174.5, 163.5, 137.6, 127.7, 121.6, 120.6, 115.2, 59.5, 51.7, 50.0, 36.0, 35.5, 34.5; MS (electrospray ionization, MeOH) m/e 352 ($M+H^+$ for $C_{14}H_{16}N_5O_6$).

Preparation of Anhydride 5

NMR Scale. The diacid **9** (5 mg) was dissolved in d_6-acetic anhydride (0.5 ml). This solution was monitored by NMR periodically; after 5 h at room temperature, the starting diacid was completely converted to a single product whose NMR spectrum was that corresponding to anhydride **5**.

Preparative Scale. The diacid **9** (12 mg, 0.022 mmol) was dissolved in acetic anhydride (1.0 ml) and stirred under argon for 3 h. The volatiles were removed *in vacuo*; the oily residue was triturated several times with dry toluene, then with dry diethyl ether, and vacuum dried to afford the anhydride **6** (10 mg, 100%) as an oily solid that was used without further purification for conjugation experiments. ^{1}H NMR (CD_3CN) δ 8.0 (d, 6 Hz, 1H), 7.2–7.4 (m, 5H), 4.7 (m, 1H), 3.8 (s, 3H), 3.7 (s, 3H), 3.2 (dd, J = 12, 5 Hz, 2H), 3.0 (dd, J = 12, 3 Hz, 2H).

Preparation of [Bis-(2-*N*-methylimidazolyl)methanol] Copper(II) Dichloride 6-$CuCl_2$. A 10 mM solution of the bis-imidazole carbinol 6 in acetonitrile was added dropwise to an equal volume of 10 mM $CuCl_2 \cdot 6H_2O$ in acetonitrile resulting in an immediate color change to greenish blue and gradual crystallization of blue L·$CuCl_2$ (**7**) in nearly quantitative yield. The crystals, which were washed with acetonitrile and dried, were suitable for x-ray diffraction. UV-Vis (CH_3CN, λ, ε) 204 (2,400), 242 (2,100), 275 (1,000), 338 (200), 600–750 (50).

Conjugation Procedures

General Method for Modification of 38C2 with Lactones 2a–2e and Anhydrides 3a–3e. In a typical assay, 38C2 (0.5 μM) was incubated with either a lactone **2a–2e** (250 μM) or anhydride **3a–3e** (250 μM) in PBS (pH 7.4) for 30 min at room temperature. The reaction was initiated by addition of the inhibitor from a stock solution in acetonitrile such that the organic cosolvent was equal to 5%. After 30 min, the unreacted lactones or anhydrides were removed by dialysis (10,000 molecular weight membrane for 24 h) at 4°C in 50 mM PBS (pH 7.4).

Large-Scale Preparation of 38C2–5-$CuCl_2$

Method A. In a representative experiment, a solution of 38C2 (2.85 ml, 30 μM) in 50 mM NaCl (pH 5.8) was treated with a solution of **5** (150 μl, 40 mM) in DMSO and allowed to stand at room temperature overnight. This solution was then treated with 150 μl of $CuCl_2$ (40 mM) in DMSO and allowed to stand overnight at room temperature. The resulting solution was then dialyzed (10,000 molecular weight membrane for 24 h) at 4°C in 50 mM NaCl (pH 5.5).

Method B. In a representative experiment, a solution of **5**-$CuCl_2$ was prepared by addition of **5** (25 μl, 40 mM) in DMSO to $CuCl_2$ (25 μl, 40 mM) in DMSO. The resulting blue-green solution was added to 38C2 (2.0 ml, 50 μM) in 50 mM NaCl and allowed to stand at room temperature overnight. The resulting solution was then dialyzed (10,000 molecular weight membrane for 24 h) at 4°C in NaCl (50 mM, pH 5.5).

Samples for inductively coupled-plasma-MS Cu-analysis were prepared by dialyzing solutions of the proteins into doubly distilled water (1:200 vol/vol) to remove interfering sodium ion. Analysis of 38C2-**5**-$CuCl_2$ (2.9 mg/ml) gave 310 μg of Cu/liter

(≈0.25 Cu/Ab); analysis of 38C2 (2.9 mg/ml, no Cu added) showed 13 μg of Cu/liter (≈0.01 Cu/Ab).

Steady State Kinetic Experiments

Allylic Rearrangement of (*S*)-(+)-4a to Enone 4b. The assays were performed as originally described (51). The assay was initiated by addition of **4a** in acetonitrile (reaction concentration 250 μM) to a thermostated (23°C, ±1°C) cuvette containing either buffer (PBS, pH 7.4), 38C2 (0.5 μM in PBS, pH 7.4), or covalently modified 38C2 (0.5 μM in PBS, pH 7.4). Assays were performed with UV spectrophotometry; the assay volume was 1 ml, and the organic cosolvent was 5% by volume. The assay was conducted by observing the increase in absorbance because of the formation of enone **4b** (ε_{248} = 15,120 $M^{-1}\cdot cm^{-1}$) on a Shimadzu UV/vis spectrophotometer. Experiments were performed in duplicate, and the reaction was followed for no more than 5% of the reaction, during which time the reaction progress was linear ($r^2 > 0.985$).

Time Course of the Modification of Antibody 38C2 with 3e in the Presence and Absence of 2,4-Pentanedione. Modification conditions: [38C2] 2 μM, [**3e**] 200 μM; buffer conditions: 5% CH_3CN and 95% PBS (pH 7.4). The activity of the modified 38C2 was measured by removal of aliquots at time *t* during the assay and determination of the initial rate of isomerization of **4a** as detailed above. Note that **3e** had no effect on the rate of the isomerization reaction. The modification was also performed in the presence of 2,4-pentanedione (200 μM) under the same conditions.

Kinetic Parameter Measurement of 38C2–5-$CuCl_2$ Catalyzed Hydrolysis of Ester 11. Hydrolysis of **11** was assayed in 4-morpholinepropanesulfonic acid (Mops) buffered solutions (25 mM, 25 mM NaCl, pH 7.0) with spectrophotometric monitoring of the product 4-nitrophenolate at 400 nm in the presence or absence 38C2-**5**-$CuCl_2$ (1 μM). The reaction was initiated by addition of stock solutions of the freshly prepared substrate **11** in dry DMSO to the catalyst solution to give substrate concentrations from 100 to 500 μM. Experiments were performed in duplicate, and the reaction was followed for no more than 5% of the reaction, during which time the reaction progress was linear ($r^2 > 0.985$). Kinetic parameters were determined by fitting the initial rate data to the Michaelis–Menten model with the ENZFITTER computer program.

The k_{uncat}(**11**) rate constant for the uncatalyzed hydrolysis of **11** was measured by using the method of initial rates. The reactions were performed in duplicate in an ELISA microtiter plate and were initiated by addition of the substrate **11** into buffer (25 mM Mops, 25 mM NaCl, pH 7.0) at 23°C to give final concentrations in the range of 50–500 μM. The hydrolysis of **11** was monitored by formation of *p*-nitrophenolate at 400 nm. The reaction was sufficiently slow to require sealing of the ELISA plate to prevent evaporation. The reaction was followed for no more than 5–10% of reaction, during which the progress curves were linear ($r^2 > 0.985$). The k_{uncat} was determined graphically by plotting the rate of phenolate formation against [**11**]. There was no measurable buffer effect on the noncatalyzed rate of hydrolysis of **11**.

Measurement of the Bimolecular Rate Constant *k* for the Reaction Between 6-$CuCl_2$ and 11. The reaction between **6**-$CuCl_2$ and **11** was followed by the method of initial rates under pseudo-first-order assay conditions. The concentration of **11** was fixed at three concentrations in excess (300, 400, and 500 μM) in Mops buffer (25 mM, 25 mM NaCl, pH 7.0). The concentration of complex **6**-$CuCl_2$ was varied (15, 30, 45 μM). The reaction was followed for <5% conversion, during which the rates were linear ($r^2 >$ 0.985). The bimolecular rate constant *k* (**6**-$CuCl_2$) was determined by a two-step graphical process. First, the observed rate k_{obs} (at each fixed concentration of **11**) was determined from a plot of rate vs. [**6**-$CuCl_2$] (GRAPHPAD PRISM software). The k_{obs} values where then plotted vs. [**11**] to give the true rate constant *k* (**6**-$CuCl_2$) = 0.03 $M^{-1}\cdot min^{-1}$.

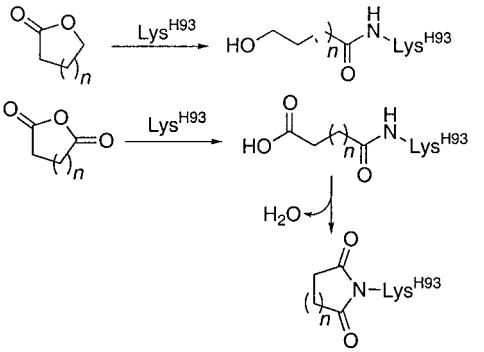


Fig. 1. Potential mechanisms of covalent modification of Lys^{H93} of 38C2 by either lactones or anhydrides.

Results and Discussion

A previous approach to covalent modification of Lys^{H93} of 38C2 used *N*-acyl β-lactam derivatives (48). The authors noted that the reactivity of the antibody with these electrophiles was low, and that the time taken for efficient derivatization ranged from 3 to 40 h. Therefore, we first sought to develop a reagent for covalent modification of Lys^{H93} that would be more reactive than *N*-acyl β-lactams, to optimize selectivity and minimize surface modification. The only limitations were that it should be stable in aqueous buffer, and it must form an essentially irreversible linkage to Lys^{H93}. For these reasons, we primarily explored the ability of a range of lactones (**2a–2e**) and anhydrides (**3a–3e**) to modify 38C2 (Figs. 1 and 2).

Modification of the antibody active site was followed in two ways. The binding-site Lys^{H93} was titrated with pentane-2,4-dione and followed by spectroscopic determination of the enaminone absorbance at 318 nm throughout the incubation (46, 47). In addition, the initial rate of 38C2-catalyzed isomerization (51) of (*S*)-(+)-β,γ-unsaturated ketone **4a** to α,β enone **4b**

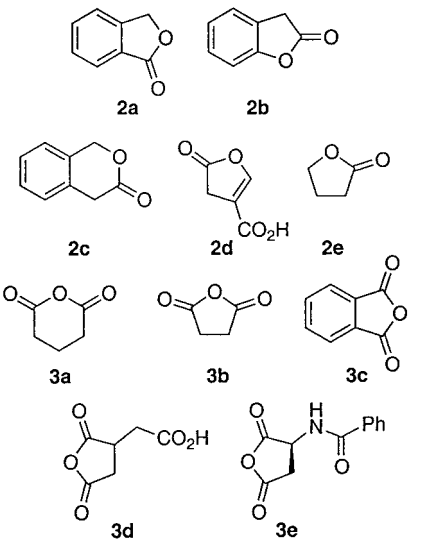
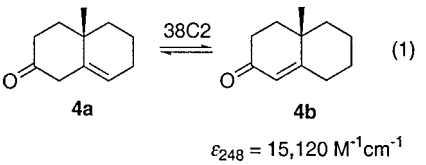


Fig. 2. Lactones **2a–2e** and anhydrides **3a–3e** used as covalent modifying agents of 38C2.

was measured at times throughout the conjugation process (Scheme 1).



Scheme 1.

In a typical modification experiment, the labeling reagent (**2a–2e**) and (**3a–3e**) (250 μM) was incubated with 38C2 (0.5 μM) in PBS (pH 7.4) at room temperature for 30 min. The inhibition of the initial rate of 38C2 formation of (*S*)-(+) **4b** was then determined spectrophotometrically (248 nm) by addition of octalone **4a** (250 μM). The data for the panel of lactones (**2a–2e**) and anhydrides (**3a–3e**) are shown in Table 1.

The lactones, in general, are much less effective derivatizing agents of 38C2 than their anhydride counterparts. As an example, γ-butyrolactone **2e** inhibits the activity of 38C2 by only 1.5%, whereas its structural homologue succinic anhydride **3b** knocks out 97.4% of the 38C2 activity; isophthalide **2a** inhibits the 38C2-catalyzed process by only 1.6%, whereas phthalic anhydride **3c** inhibits 98.4% of the catalytic rate. Interestingly, the original report of covalent modification of 38C2 noted that *N*-acyl γ-lactams, the lactam structural analog of **2e** and **3c**, do not react with 38C2 at all. In addition, the anhydride motif, regardless of the additional structural constituents of the inhibitor, seems to be fairly generic and well tolerated by 38C2. All of the anhydrides **3a–3e** reduce the activity of 38C2 by >95% after 30-min exposure. Furthermore, the lactone-mediated inhibition of 38C2 exhibits strict structural requirements. For example, 2-coumaranone **2b** is a 93.7% inhibitor, whereas the isomeric isophthalide **2a** is only a 1.6% inhibitor. On the basis of these results, we have shown that anhydrides are, by far, the most efficient and general derivatizing agents for the 38C2 antibody yet reported.

Table 1. Covalent modification of 38C2 by lactones 2a–e and anhydrides 3a–3e

Electrophile	Inhibition*, %
2a	1.6
2b	93.7
2c	22.7
2d	7.4
2e	1.5
3a	98.9
3b	98.3
3c	97.4
3d	98.4
3e	95.6

In a typical assay, 38C2 (0.5 μM) was incubated with either the lactone **2a–e** (250 μM) or anhydride **3a–e** (250 μM) in PBS (pH 7.4) for 30 min at room temperature. The reaction was initiated by addition of the inhibitor from a stock solution in acetonitrile such that the organic cosolvent was equal to 5%.

*Inhibition of the 38C2-catalyzed production of enone **4b** from **4a** was monitored spectrophotomerically by the increase in absorbance at 248 nm (Scheme **1**). Inhibition was determined from the difference in the initial rates of 38C2-catalyzed formation of **4b** in the presence and absence of inhibitor and the background reaction and converted to a percentage.

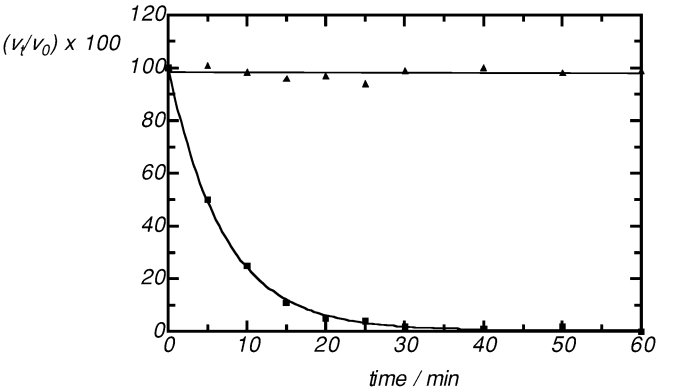


Fig. 3. Time-dependent inhibition of 38C2 by anhydride **3e** in the presence (▲) and absence (■) of pentane-2,4-dione. V_o = initial rate at t = 0; V_t = initial rate after time t of inhibition.

In-depth kinetic studies revealed that the reaction of anhydride **3e** is time-dependent and can be quantitatively inhibited by addition of pentane-2,4-dione (Fig. 3).

Although Lys^{H93} is by far the most likely binding-site residue to be modified during the reaction, it is feasible that the actual target could be an alternative binding-site residue such as Tyr^{L41}. In such a case, the failure to form the enamine complex between Lys^{H93} and pentane-2,4-dione would simply be a result of steric hindrance within the antibody-combining site. To investigate whether Tyr^{L41} is being acylated, **3e**-modified 38C2 (>98%, 20 μM) was treated with hydroxylamine (200 mM) for 24 h at room temperature. After dialysis (to remove the excess hydroxylamine), titration of Lys^{H93} with 2,4-pentanedione revealed no further enamine complex formation, supporting the notion that Lys^{H93} is indeed the target for the anhydride-modifying agents.

Having thus revealed anhydrides to be excellent and generic derivatizing agents for 38C2, we then synthesized a first-generation bis-imidazolyl aspartic acid anhydride derivative **5** (Fig. 4).

The known bis-imidazolyl carbinol **6** was attached to an aspartic acid anhydride tether via a nucleophilic reaction with

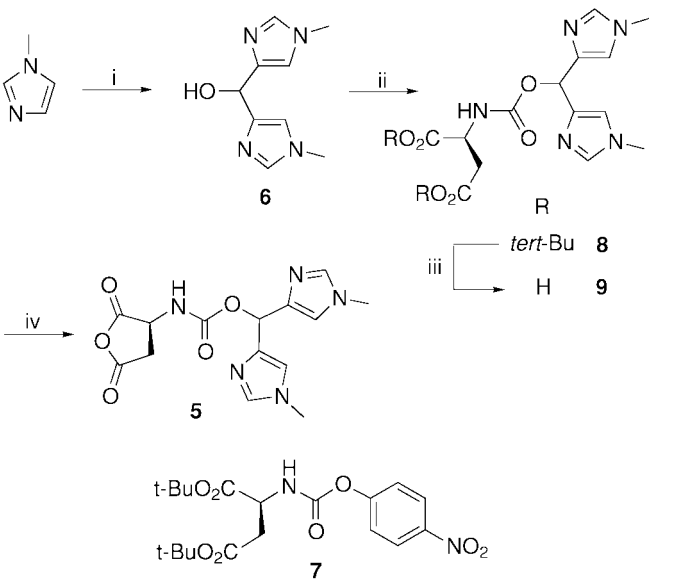


Fig. 4. Reagents and conditions: (*i*) *n*-BuLi; HCO_2Bu; H_3O^+; (*ii*) NaH; **7**; (*iii*) trifluoroacetic acid/$CH2Cl_2$; (*iv*) Ac_2O.

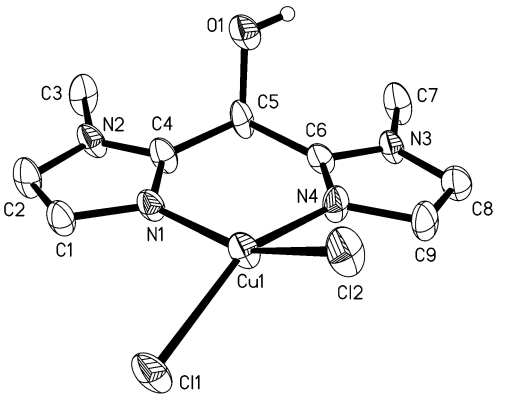


Fig. 5. X-ray (ORTEP) structure of **6**-$CuCl_2$.

the key carbamate ester derivative **7** (68%), prepared in one step from di-*tert*-butyl aspartate and 4-nitrophenyl chloroformate (90%). Conversion of the resulting carbamate di-*tert*-butyl ester **8** to the diacid **9** was achieved by careful trifluoroacetic acid protonolysis (100%). The diacid was then quantitatively converted into anhydride **5** by dehydration with acetic anhydride.

The tight association of Cu(II) with the bis-imidazole intermediates **5–8** was indicated by: (*i*) spectral titration of aqueous $CuCl_2$ and **6** (1:1; 280 nm, 580 nm); (*ii*) the preparation and x-ray crystallographic determination of the $CuCl_2$ complex of **6** (Fig. 5) (the x-ray crystallographic data are published as supporting information on the PNAS web site, www.pnas.org); and (*iii*) the emerald green color rapidly produced on addition of $CuCl_2$ to the imidazolium salts **8** and **9** in DMSO.

The ability of **5** to covalently modify antibody 38C2 was then investigated in two orthogonal ways to probe whether the modification of 38C2 by **5** or **5**-$CuCl_2$ might exhibit different profiles (Fig. 6). In method A, the antibody (20–30 μM) was first incubated with the cofactor ligand **5** (250 μM) in PBS (20 mM, pH 7.4) with 5% acetonitrile as cosolvent at room temperature; the reaction was typically complete after 1–3 h. Cu(II) insertion into the apo-38C2-**5** conjugate was achieved by initial dialysis into aqueous NaCl (50 mM, pH 5.5) solution followed by addition of excess $CuCl_2$ (40 mM) in DMSO (5%), and subsequent dialysis into aqueous NaCl. Metal salt addition at lower pH was necessary to minimize precipitation of hydroxo-Cu(II) species.

In method B, **5** was precoordinated with $CuCl_2$ (in DMSO), then the resulting blue-green **5**-$CuCl_2$ complex was incubated with 38C2 in aqueous NaCl (3 h) and finally dialyzed in NaCl. The incorporation of Cu(II) into the 38C2-**5** conjugate was confirmed by inductively coupled-plasma-MS.

The 38C2-**5**-$CuCl_2$ metalloantibody complex was then tested for its ability to catalyze either oxidation or acyl transfer reactions. No catalysis by 38C2-**5**-$CuCl_2$ was observed for the

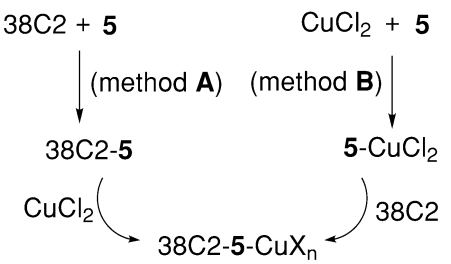


Fig. 6. Orthogonal approaches to covalent modification of 38C2.

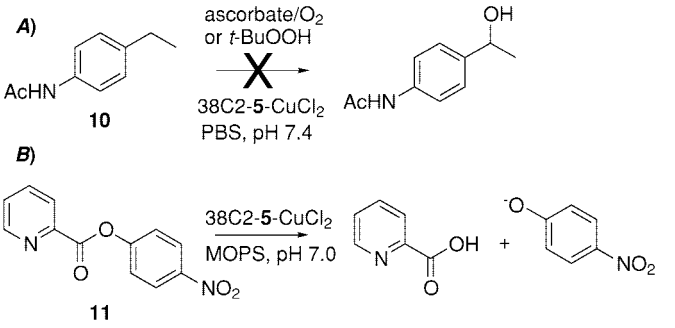


Fig. 7. (*A*) Attempted benzylic oxidation of substrate **10** by 38C2-**5**-$CuCl_2$; (*B*) hydrolysis of picolinic acid ester 11 by 38C2-**5**-$CuCl_2$.

hydroxylation of a representative benzylic substrate (**10**) with either oxygen/ascorbate or *t*-BuOOH as the stoichiometric oxidant (Fig. 7*A*). However, significant and metal-dependent catalysis of hydrolysis of the coordinating picolinate ester **11** (52, 53) by 38C2-**5**-$CuCl_2$ in aqueous buffer [Mops (25 mM), pH 7.0] is observed (Fig. 7*B*).

The catalytic hydrolysis of **11** by the 38C2-**5**-$CuCl_2$ complex follows Michaelis–Menten kinetics [k_{cat}(**11**) = 2.3 min^{-1} and K_m(**11**) 2.2 mM). The uncatalyzed rate of hydrolysis of **11** under the conditions of our assay (in the absence of antibody and $CuCl_2$) is 1.1×10^{-5}·min^{-1}. This gives an enhancement ratio [k_{cat}(**11**)k_{uncat}(**11**)] of 2.1×10^5 and reveals the significant catalytic power of the 38C2-**5**-$CuCl_2$ conjugate. To determine the benefit of binding the Cu-bis-imidazolyl ligand into the antibody-binding site, the second-order rate constants of the modified 38C2 [k_{cat}(**11**)/K_m(**11**)], and the Cu(II)-bisimidazolyl ligand k(**6**-$CuCl_2$) = 0.03 M^{-1}·min^{-1} catalyzed processes were compared. This gives a rate enhancement {[k_{cat}(**11**)/K_m(**11**)]/k(**6**-$CuCl_2$)} of 3.5×10^4 and an effective molarity (EM) of [k_{cat}(**11**)/k(**6**-$CuCl_2$)] 76.7 M. This EM compares favorably with other antibody-catalyzed bimolecular processes (5, 6) and suggests that there is a significant benefit to binding the bisimidazolyl ligand into 38C2, and that the catalysis arises from sequestration of the substrate in proximity to the metal–ligand complex. The key role for copper in the hydrolysis of the picolinate ester substrate **11** is indicated by the nearly complete inhibition (>95%) of the **6**-$CuCl_2$ and 38C2-**5**-$CuCl_2$ catalyzed reactions by addition of EDTA (3–5 μM).

In conclusion, we have shown that anhydrides are reactive and selective generic derivatizing agents for the Lys^{H93} residue of the 38C2 antibody. In addition, we have successfully derivatized the antibody 38C2 with a biomimetic bis-imidazolyl metal-binding cofactor **5** and have demonstrated that the resultant copper-complexed antibody possesses significant and metal-dependent esterase activity. This is, in fact, the first such example, to our knowledge, of a copper-dependent catalytic antibody and replaces the original aldolase function of the protein with a new hydrolytic function. In addition to establishing the viability of our cofactor approach to elicit efficient catalytic metalloantibodies, these results bode well for the development of new semisynthetic metalloproteins that combine the unique selectivity of natural enzymes with the broader substrate specificity of synthetic catalysts.

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