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RADIOLABELING OF PROTEINS WITH RADIOISOTOPES OF COPPER USING p-CARBOXYALKYLPHENYLGLYOXAL bis-(N^4-METHYLTHIOSEMICARBAZONE) (TSC) BIFUNCTIONAL CHELATES

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SUMMARY

A series of p-carboxyalkylphenylglyoxal and p-carboxyalkyl-1,2-diketo-bis-(N^4-methylthiosemicarbazone) bifunctional ligands (TSC) have been prepared and evaluated for use in binding radioisotopes of copper to antibodies. We have developed an improved synthesis of the requisite α-ketoaldehyde and 1,2-diketone substrates used for derivatization to the bis-TSC bifunctional chelates. This approach utilizes a modified Kornblum method and provides a simple alternative to the usual method for fabrication of the 1,2-bis-TSC ligands, which avoids the use of highly toxic selenium dioxide for oxidation of substituted acetophenones to 1,2 dicarbonyl compounds. The overall yields of the bis-TSC chelates using this procedure were 8-60%. The effects of the alkyl chain length and substitution on the C-2 position on p-carboxyalkylphenyl-1,2-diketo bis-TSC bifunctional chelate for attaching radioisotopes of copper to proteins were studied. Following complexing copper-64 or copper-67 to the bis-TSC chelate, the acid moiety of the TSC chelate was activated as the tetrafluorophenyl ester. The copper-labeled activated TSC chelate was attached to bovine serum albumin under mild conditions in 3% to 40% yield. These studies have demonstrated that the shorter chain analogues of the TSC chelates from the 1,2-diketones give the highest radiolabeling yields.

Key Words: Antibodies, Bifunctional Chelate, Bis-thiosemicarbazones, Copper Radioisotopes, Protein Radiolabeling, Tumor Imaging and Therapy.

INTRODUCTION

Current increased interest in the availability of radionuclides for therapy has resulted from the recent success and potential widespread importance of radiolabeled tumor-specific antibodies

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or antibody fragments for both diagnosis and therapy of cancer. Radiolabeled antibody technology has focused on the use of single-photon emitting radionuclides such as technetium-99m, iodine-131, iodine-123, and indium-111 because of their widespread availability and use with gamma cameras for both planar and single-photon computerized tomographic (SPECT) imaging.[1-5] Because of the much greater resolution of positron emission tomography (PET) compared with SPECT (2-5 mm vs. 10-15 mm), the potential use of antibodies labeled with positron-emitting radionuclides for radioimmunodetection is also of great interest. A limited number of radionuclides, however, have the optimal combination of availability and chemical and radionuclidic properties required for diagnostic applications with PET. One method for labeling antibodies involves direct attachment of the radioisotope to an amino acid residue of the protein. With direct radiolabeling using radioiodine and technetium-99m, for example, the radionuclide can often become easily detached from the antibody. For many other radionuclides such as copper and indium, however, the direct radiolabeling approach cannot be used and use of bifunctional chelate techniques are required for complexation of the radioisotope to the antibody. This approach allows the development of chelating agents designed to bind radioisotopes in a way stable to physiological conditions.

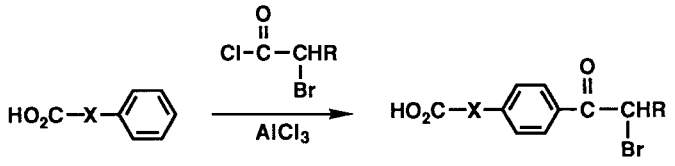
Because of the time period normally required for vascular clearance and tumor-specific surface antigen binding, imaging of tumors is usually not possible until 12 to 48 hours following intravenous administration of radiolabeled antibodies. There are only a few positron-emitting radionuclides that have a sufficiently long physical half-life, are readily available, and have a high enough positron yield for radioimmunodetection (Table 1). Recent protein radiolabeling studies with positron-emitting radionuclides have focused on the use of fluorine-18 (F-18). Despite the relatively short 110-min half-life, the ready availability of F-18 from medical cyclotrons and the broad spectrum of chemical techniques which are now available for introduction of this

radionuclide into organic molecules have stimulated these studies. The formation of 4-[^{18}F]fluorophenacyl bromide and 3-[^{18}F]fluoro-5-nitrobenzimidate have been reported,[6] and the N-(p-[^{18}F]-fluorophenyl)maleimide agent has been used to radiolabel the Fab' fragment of IgG.[7]

Table 1. Potential Positron-Emitting Radionuclides for Protein Radiolabeling

Radioisotope	Half-Life	Positron Energy (MeV)	Percent Abundance	Production Mode
		Accelerator Produced		
Ga-68	1.1 h	1.89	90	^{68}Ge/^{68}Ga generator
Br-75	1.6 h	1.74	75	^{79}Br(p,5n)^{75}Kr→^{75}Br
F-18	1.8 h	0.64	100	^{18}O(p,n)^{18}F
Cu-61	3.4 h	1.20	62	^{59}Co(α,2n)^{61}Cu
Co-55	17.5 h	1.51	77	^{58}Ni(p,α)^{55}Co ^{56}Fe(p,2n)^{55}Co
As-72	26 h	7.50	75	Se-72 generator ^{75}As(p,4n)^{72}Se
Zr-89	78.4 h	0.89	22	^{89}Y(p,n)^{89}Zr
		Reactor Produced		
Cu-64	12.7 h	0.65	18	^{63}Cu(n,γ)^{64}Cu ^{64}Zn(n,p)^{64}Cu

The 110-min half-life of fluorine-18, however, would not be expected to be optimal for the requirement of delayed imaging. As an alternative, copper-64 has the advantage of a relatively long 12.7 h half-life, which would allow the necessary time required for imaging.



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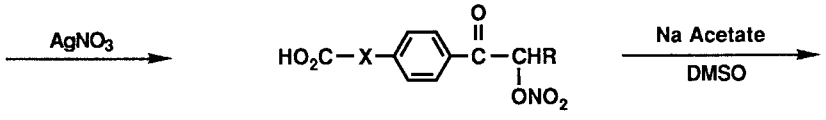
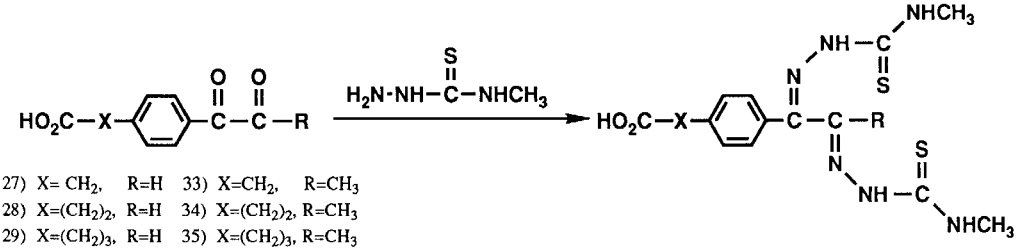
3) X=$(CH_2)_3$, R=H 9) X=$(CH_2)_3$, R=CH_3
4) X=$(CH_2)_4$, R=H 10) X=$(CH_2)_4$, R=CH_3
5) X=$(CH_2)_5$, R=H 11) X=$(CH_2)_5$, R=CH_3
6) X=$(CH_2)_8$, R=H 12) X=$(CH_2)_9$, R=CH_3
13) X=benzyl, R=CH_3



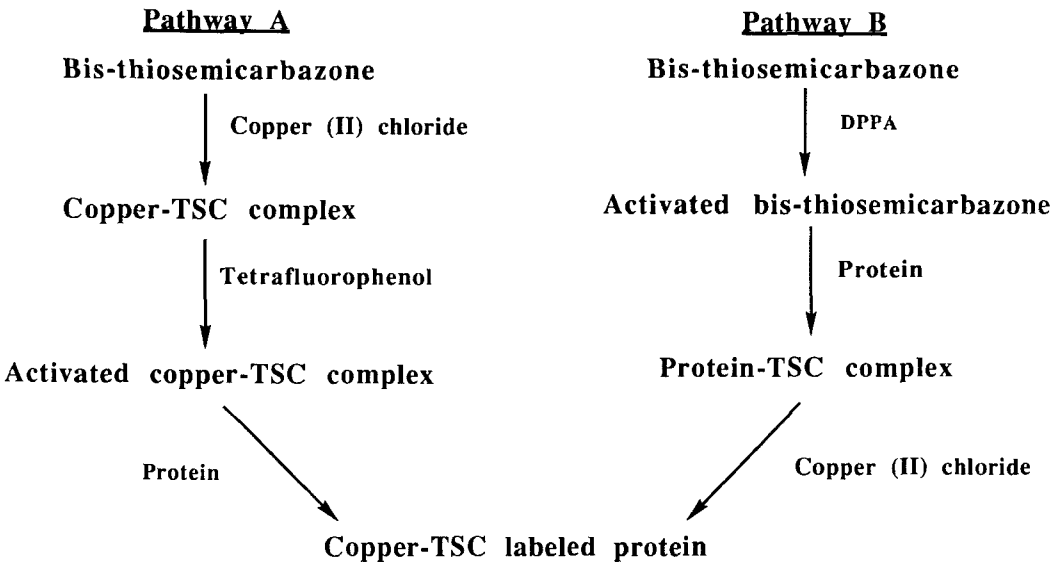
14) X= CH_2, R=H 20) X=CH_2, R=CH_3
15) X=$(CH_2)_2$, R=H 21) X=$(CH_2)_2$, R=CH_3
16) X=$(CH_2)_3$, R=H 22) X=$(CH_2)_3$, R=CH_3
17) X=$(CH_2)_4$, R=H 23) X=$(CH_2)_4$, R=CH_3
18) X=$(CH_2)_5$, R=H 24) X=$(CH_2)_5$, R=CH_3
19) X=$(CH_2)_8$, R=H 25) X=$(CH_2)_9$, R=CH_3
26) X=benzyl, R=CH_3



27) X= CH_2, R=H 33) X=CH_2, R=CH_3
28) X=$(CH_2)_2$, R=H 34) X=$(CH_2)_2$, R=CH_3
29) X=$(CH_2)_3$, R=H 35) X=$(CH_2)_3$, R=CH_3
30) X=$(CH_2)_4$, R=H 36) X=$(CH_2)_4$, R=CH_3
31) X=$(CH_2)_5$, R=H 37) X=$(CH_2)_5$, R=CH_3
32) X=$(CH_2)_8$, R=H 38) X=$(CH_2)_9$, R=CH_3
39) X=benzyl, R=CH_3

40) X= CH_2, R=H 46) X=CH_2, R=CH_3
41) X=$(CH_2)_2$, R=H 47) X=$(CH_2)_2$, R=CH_3
42) X=$(CH_2)_3$, R=H 48) X=$(CH_2)_3$, R=CH_3
43) X=$(CH_2)_4$, R=H 49) X=$(CH_2)_4$, R=CH_3
44) X=$(CH_2)_5$, R=H 50) X=$(CH_2)_5$, R=CH_3
45) X=$(CH_2)_8$, R=H 51) X=$(CH_2)_9$, R=CH_3
52) X=benzyl, R=CH_3

Scheme I. Synthesis of p-carboxyalkyl phenylglyoxals via the "Kornblum" approach.



Scheme II. Formation of Cu(II) complexes from bis-(N-alkylthiosemicarbazones).

Bis-N-alkylthiosemicarbazone bifunctional chelates have been used for radiolabeling of antibodies with technetium-99m.[8,9,10] The reaction conditions employed for the attachment of technetium to the antibody, however, are harsh and can result in loss of immunoreactivity. The binding of copper to bis-TSC ligands is well known and proceeds in high yield under mild conditions.[11] Monofunctional copper-TSC complexes have been shown to be relatively stable in vivo over a short period of time ($\sim$ 2 h)[14] and may therefore have potential for the radiolabeling of antibodies. Approaches that have been reported for radiolabeling proteins with radioisotopes of copper include the use of the 6-p-aminobenzyl-TETA chelate for attaching copper-67 for radiolabeling of Lym-1, a monoclonal antibody against human B cell lymphoma.[15] The use of macrocyclic amine chelating agents for attachment of copper-64 to antibodies has also been described.[16] The use of the bis-TSC bifunctional chelates approach offers a number of advantages

over these techniques due to the ease of formation of the copper-bis-TSC chelate and stability of these copper(II) complexes.[17] We report the preparation of a series of bis-N-alkylthiosemicarbazones (Scheme I) and labeling studies with bovine serum albumin (BSA) and IgG (Scheme II). The effect of alkyl substitution on the C-2 position and alkyl chain length on the labeling efficiency of proteins with radioisotopes of copper has been investigated.

CHEMISTRY

The bifunctional bis-TSC chelates were prepared as shown in Scheme 1. The reported synthesis of these ligands involves the use of selenium dioxide in the oxidation of the methyl ketone to afford the desired 1,2-diketo product.[8-11,18] However, in addition to the extreme toxicity of selenium dioxide, the reaction yields are often low and purification of the products is difficult. An improved synthesis for these ligands was developed utilizing a modified procedure of Kornblum and Frazier.[19] The α-bromo ketone intermediate was prepared by the Friedel-Crafts acylation of p-phenylalkanoic acid with either bromoacetyl chloride (**1-6**) or 2-bromopropionyl chloride (**7-13**). The α-bromo ketones were then treated with a slight excess of silver nitrate to afford the desired nitrate esters (**14-26**). A sodium acetate catalyzed oxidation-elimination in dimethyl sulfoxide was carried out on the nitrate esters to afford the 1,2-diketo compounds (**27-39**) (Table 2). Condensation of the 1,2-dicarbonyl compounds with an excess of the bis-(4-methyl-3-thiosemicarbazide) afforded the desired bis-TSC chelates in good yield using HCl as a catalyst (Table 3). Treatment of compounds **40-52** with cupric (II) acetate in acetate buffer (pH 3.1) readily afforded the copper chelates under mild conditions. The copper complexes were identified by the characteristic shift in the ultraviolet region of the absorption maxima from 340 nm to 310 nm in addition to a new absorption in the visible region (550 nm) as shown in Table 4.

Table 2. Property of α-Bromoketones (**1**→**13**), Nitrate Esters (**14**→**26**), α-Ketoaldehydes (**27**→**32**), and Diketones (**33**→**39**).

α-Bromo Ketones			Nitrate Esters[a]		α-Ketoaldehydes and Diketones		
Compound	% Yield	mp (°C)	Compound	% Yield	Compound	% Yield	mp (°C)
1	68 white solid	103-105	**14**	86 pale white solid	**27**	67 yellow solid	b
2	40 white solid	148-149	**15**	94 white solid	**28**	97 pale yellow solid	b
3	62 white solid	85-86	**16**	92 white solid	**29**	82 orange oil	...
4	80 white solid	112-113	**17**	93 white solid	**30**	100 orange oil	...
5	76 yellow solid	62	**18**	70 pale yellow solid	**31**	89 orange oil	...
6	20 white solid	98	**19**	75 pale yellow oil	**32**	100 orange oil	...
7	27 yellow solid	144-146	**20**	89 white solid	**33**	70 yellow solid	101
8	61 white solid	137-138	**21**	100 yellow oil	**34**	68 yellow solid	73
9	62 white solid	66	**22**	100 yellow oil	**35**	72 yellow oil	...
10	74 white solid	100-102	**23**	95 white solid	**36**	55 yellow solid	100
11	82 white solid	65-66	**24**	83 white solid	**37**	97 yellow oil	...
12	78 white solid	119	**25**	83 yellow oil	**38**	70 yellow oil	...
13	66 white solid	151	**26**	81 white solid	**39**	58 pale yellow solid	153

[a]The melting points of the nitrate esters were not determined since these intermediates were not purified and were used directly for the next reaction.

[b]Not determined.

Table 3. Properties of N-Methylthiosemicarbazone Derivatives.

Compound	% Yield		mp (°)	analysis[b]
40	36 (14)[a]	orange yellow solid[c]	202-204	C,H,N,S
41	42 (16)[a]	orange yellow solid	190-193[d]	C,H,N,S
42	57 (27)[a]	orange yellow solid[c]	205-206	C,H,N,S
43	52 (40)[a]	orange yellow solid[c]	175-178	C,H,N,S
44	42 (21)[a]	yellow solid[c]	162-165	C,H,N,S
45	52 (8)[a]	yellow oil		C,H,N,S
46	59 (10)[a]	yellow solid[e]	202	C,H,N,S
47	67 (29)[a]	pale yellow solid[e]	218	C,H,N,S
48	66 (30)[a]	yellow solid[e]	193-194	C,H,N,S
49	54 (22)[a]	yellow solid[e]	189-190	C,H,N,S
50	93 (63)[a]	yellow oil		C,H,N,S
51	95 (44)[a]	yellow oil		C,H,N,S
52	61 (19)[a]	yellow solid[d]	202	C,H,N,S

[a]yield from phenylalkanoic acid
[b]samples contain 0.25-1.5 mol ethanol
[c]from ethanol water
[d]literature 216-217
[e]from methanol water

Table 4. Ultraviolet Properties of Cu(II) bis-TSC Complexes.

Compound	R	X	ϵ(free ligand) (nm)	ϵ(copper complex) (nm)
40	H	CH_2	6110 (340)	6110 (312) 2550 (500)
41	H	$(CH_2)_2$	6220 (340)	6330 (308) 2760 (500)
42	H	$(CH_2)_3$	6070 (340)	6280 (310) 2830 (500)
43	H	$(CH_2)_4$	5160 (340)	6120 (310) 2350 (500)
44	H	$(CH_2)_5$	6130 (340)	5900 (308) 1900 (500)
45	H	$(CH_2)_8$	4600 (340)	4700 (308) 2100 (492)
46	CH_3	CH_2	7000 (348)	6400 (310) 3100 (490)
47	CH_3	$(CH_2)_2$	6500 (348)	5800 (310) 2800 (490)

Table 4. Ultraviolet Properties of Cu(II) bis-TSC Complexes.

Compound	R	X	ϵ(free ligand) (nm)	ϵ(copper complex) (nm)
48	CH_3	$(CH_2)_3$	6500 (348)	6200 (310) 2900 (490)
49	CH_3	$(CH_2)_4$	6600 (348)	5900 (310) 2700 (490)
50	CH_3	$(CH_2)_5$	6500 (348)	5400 (310) 1870 (488)
51	CH_3	$(CH_2)_9$	6700 (332)	5900 (312) 2100 (490)
52	CH_3	$-CH_2-C_6H_4-$	6300 (328)	6400 (312) 2700 (490)

RADIOCHEMISTRY

The method utilized for the labeling of BSA and IgG with radioisotopes of copper is shown is Scheme II. Utilizing pathway A, the bis-TSC chelate was stirred with either copper-64 or copper-67 of varying specific activity at 70°C in 0.2M acetate buffer (pH 3.1) for 15 min to afford the copper-bis-TSC complex. Unbound copper was removed by purification using a RP-C18 Sep Pak. The acid moiety was converted to the tetrafluorophenol ester by reacting the complex with tetrafluorophenol and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in aqueous acetonitrile (pH 6.0) at 70°C for 30 min. Purification of the activated copper-bis-TSC complex using a RP-C18 Sep Pak allowed for the removal of copper which may become unbound under the reaction conditions. The activated copper-bis-TSC complex was then added to a solution of protein in 0.2M phosphate buffer (pH 9.0) and the solution was gently stirred at room temperature for 15 min. The reaction was stopped by the addition of lysine and the copper-labeled protein was purified by passage through a Sephadex G-25 column. The results from this study are shown in Table 5. The large deviations observed in the data shown in Table 5 are due to the poor solubility of the bis-TSC ligands in the solvents employed to couple copper to the protein. The method utilized for the labelling of IgG was similar to above except that the protein activated copper-ligand complex was stirred at 37°C for 30 minutes. The percent of copper bound to IgG was determined by ITLC analysis using 0.1M $NaHCO_3$ as the mobile phase.

Table 5. Radiochemical Yield (N ≥ 2) for Binding Copper to BSA

Compound	Copper Binding	Esterification	Protein Binding	Overall
40	85.5 (± 2.1)	56.8 (± 11.0)	26.9 (± 17.2)	12.0 (± 5.7)
41	75.5 (± 23.4)	71.3 (± 17.9)	33.6 (± 19.6)	16.4 (± 7.5)
42	64.2 (± 25.9)	56.5 (± 6.4)	36.2 (± 27.0)	11.5 (± 8.1)
43	57.8 (± 27.1)	54.2 (± 12.2)	34.8 (± 7.9)	9.8 (± 1.7)
44	69.5 (± 10.0)	41.8 (± 9.3)	26.7 (± 22.9)	10.2 (± 9.4)
45	27.0 (± 18.4)	30.5 (± 9.2)	55.7 (± 33.0)	3.7 (± 1.6)
46	74.0 (± 16.4)	66.7 (± 10.1)	31.0 (± 1.3)	12.8 (± 0.6)
47	79.7 (± 17.3)	60.7 (± 11.3)	61.6 (± 23.3)	32.6 (± 9.1)
48	80.0 (± 24.2)	54.2 (± 6.7)	49.9 (± 2.8)	21.0 (± 9.5)
49	58.6 (± 21.1)	59.0 (± 13.1)	46.6 (± 13.4)	14.5 (± 5.7)
50	56.1 (± 11.7)	48.7 (± 14.8)	50.4 (± 41.3)	14.7 (± 11.7)
51	30.6 (± 0.8)	46.5 (± 12.1)	52.6 (± 26.3)	7.0 (± 2.1)
52	50.9 (± 7.1)	65.3 (± 2.5)	46.0 (± 40.3)	13.2 (± 9.3)

As shown in pathway B, compound **47** was bound to IgG by the phosphoryl azide method[9] in a 1:1 molar ratio as determined by UV/visible analysis. The IgG-bis-TSC ligand complex was then stirred at 37°C in the presence of copper-67 followed by purification utilizing a Sephadex G-25 column. The percent of copper bound covalently to the protein was determined by ITLC as above.

RESULTS AND DISCUSSION

Our interest has focused on the potential use of copper-64 for labeling tumor-specific antibodies for imaging by PET. The attractive feature for the use of copper-64 is its relatively long physical half-life (12.7 h) which is compatible with the usual prolonged period between administration and imaging (24 to 48 h) required to allow vascular clearance and tumor specific uptake. Another isotope of copper which is of interest is copper-67 which emits a high-energy beta particle which can be employed for the treatment of cancer.[24] Copper-64 is reactor produced via the copper-63 (n,γ) copper-64 reaction and can be produced with high specific activity. For example, the saturation yield at a thermal neutron flux of 2.5 x 10^{15} n·cm^2/s produces copper-64 with a specific activity of ~ 2 Ci/mg. Carrier-free copper-64 can also be reactor produced by the zinc-64 (n,p) copper-64 reaction in reactors with high epithermal neutron flux. An additional attractive feature is that the zinc and/or copper targets can be easily processed at low cost.[25]

1,2-Bisthiosemicarbazones ligands are known to strongly bind copper (II) to form stable neutral complexes. Crystallographic studies have demonstrated these complexes have a square planar N_2S_2 structure.[20] Bis-TSC-copper complexes have been prepared of simple monofunctional complexes such as 3-ethoxy-1-oxobutyraldehyde, and have been shown to exhibit antitumor effects.[21] Other investigators have recently described the synthesis of simple, monofunctional bis-TSC copper(II) complexes to study cerebral and myocardial blood flow with copper-62.[14] In addition, tissue distribution studies[12,13] may support that copper-bis-TSC complexes are relatively stable under physiological conditions for approximately 2 h. Technetium-99m labeled bis-TSC bifunctional ligands have been shown to label antibodies with minimum loss of immunoreactivity[8] and have also been used in the studies of myocardial accumulation,[22] and cholescintgraphy.[23]

The reported preparation of bis-TSC bifunctional chelates involves the synthesis of α-ketoaldehydes via selenium dioxide oxidation of methyl ketones. Because of the extreme toxicity of selenium dioxide and the low yield of product obtained due to the decomposition of the α-ketoaldehyde under the harsh reaction and purification conditions, we developed an alternative synthesis to prepare the desired α-ketoaldehydes. This method is shown in Scheme I and involves the Friedel-Crafts acylation of the phenylalkanoic acid with bromoacetyl chloride. The resulting α-bromo ketone is reacted with excess silver nitrate to afford the nitrate ester in high yield. Base-catalyzed elimination with sodium acetate in DMSO at ~ 5°C then provides the desired α-ketoaldehyde. Using this approach the reaction is performed employing mild conditions, and the α-ketoaldehyde can be easily isolated from the reaction mixture and used without further purification. The α-ketoaldehyde is then condensed with 4-methyl-3-thiosemicarbazide utilizing an acid catalyst to afford the desired bis-thiosemicarbazone. Using this procedure we were also able to synthesize a series of 1,2-diketones and their subsequent bis-TSC chelates in which we changed R from a hydrogen to a methyl group (Scheme I). The overall yield of bis-TSC bifunctional chelate from the starting phenylalkanoic acid ranged from 8% to 60%. These bis-TSC chelates were complexed with copper readily in acetate buffer at room temperature as shown by the ultraviolet spectroscopy.

Evaluation of models indicates that replacement of the hydrogen at the 2-position of the 1,2-diketo compound with an alkyl group results in restriction of rotation of the thiosemicarbazone ligands and therefore may force the chelating groups into a more favorable position for copper binding. No difference was observed, however, in the rate of copper(II) binding to bis-TSC chelates of the 1,2-diketone and α-ketoaldehyde, which proceeded rapidly with both analogues at room temperature in acetate buffer as determined by UV spectroscopy.

A study on the effect of the alkyl chain length on the labeling efficiency of BSA with the series of bis-TSC chelates prepared was carried out utilizing copper-64 and copper-67 of varying specific activity and the results are shown in Table 5. Although copper is known to have a high

affinity for albumin,[26] we chose BSA for these preliminary studies since it was readily available. Under the purification conditions employed, we can effectively remove copper so there would be no unbound copper available for binding to BSA. In this study the binding conditions were kept constant so as the only difference in the radiolabeling yield was the alkyl chain length of the ligand and no attempt was made to maximize the labeling yields. It was observed from this study that as the alkyl chain length increases, the copper binding to the bis-TSC chelate decreases because as the alkyl chain length increases the bis-TSC chelate becomes less soluble under the reaction conditions. It was also observed when the alkyl chain length was 8 (**45**) or 9 (**51**) the binding of the activated copper-bis-TSC chelate to the protein increased slightly. This increase in binding, however, was not as great as the loss of copper binding to the bis-TSC chelate, thereby a lower overall yield of copper binding to the protein was observed. Also purification of the copper-chelate complex using a "Sep Pak" allows removal of free copper, thereby avoiding nonspecifically bound copper to the protein in the later steps of the reaction sequence. From this study we chose **47** as the candidate for future protein labeling studies due to the overall radiolabeling yield obtained.

The labeling of IgG with **47** was carried out in as shown in Scheme II, in which the acid moiety was activated as the tetrafluorophenol ester and then the activated copper-ligand complex was coupled to the protein (pathway A). The same procedure was carried out in which the acid group was not activated. The amount of copper covalently bound to the protein was determined by ITLC analysis and the results of this study are shown in Table 6. It was observed from these experiments that when the acid moiety is not activated as the tetrafluorophenol ester only ~ 6.0% of the activity is associated with the protein. ITLC analysis shows that the activity is covalently bound to the protein and is not bis-TSC-copper complex which was not effectively removed during the purification. The copper-ligand-protein complex was challenged by the incubation with an excess of ethylenediaminctetraacetic acid (EDTA) to determine the stability of the copper-protein complex. It was observed that >80% of the activity was associated with the complex and was stabile up to 1 hour in the presence of excess EDTA. However at 24 hours approximately 50% of the copper-67 had come off the protein and was now complexed to EDTA. After 48 hours the copper-67 was complexed to the EDTA and no longer bound to the protein.

Table 6. Radiochemical Yield (N = 2) for binding Copper-67 to IgG Utilizing **47**

Method	Yield (%)
Acid moiety activated	27.6
Acid moiety not activated	6.1
Protein-**47** complex	61.8
Protein	14.8 (pH 6.0)
	100 (pH 9.0)

Another route for the labeling of proteins with radioisotopes of copper that was carried out is shown in Scheme II and involves the attachment of the ligand on the protein followed by the complexation of copper (Pathway B). Problems which may be encountered by this method include the non-specific binding of copper to the protein. Copper-67 was also stirred with the protein at 37°C at pH 6.0 and 9.0 to determine the nonspecific binding of copper and the results are shown in Table 6. It was observed that as the pH of the solution decreases the amount of copper bound to the protein decreases. ITLC analysis of the solutions show that the copper is not covalently bound to the protein and readily comes off when challenged by EDTA. Copper-67 was also stirred with the protein-**47** complex at pH 6 at 37°C and these results are also shown in Table 6. As the above study demonstrated, there was minimal nonspecific binding of copper to the protein at pH 6.0. Therefore non-specifically bound copper was not expected to bind to IgG at the pH of the coupling reaction using this method to attach copper to IgG. From the results of the ITLC analysis of the solution, however, it was observed that only ~30% of the copper-67 was covalently bound to the protein-ligand complex. When challenged with EDTA, approximately 50% of the copper-67 that was covalently bound to the protein had complexed with EDTA within 24 hours.

CONCLUSIONS

This work demonstrates an improved synthesis for a series of p-carboxyalkylphenyl glyoxal and 1,2-diketone utilizing mild conditions and the subsequent preparation of the bis-(N-methylthiosemicarbazone) derivatives. The effect of the increasing methylene chain length on the copper binding and subsequent protein labeling have shown that as the alkyl chain length of the bis-TSC chelates increases, the labeling efficiency of the protein decreases and thereby the binding of copper with shorter chain analogues (**40-42**, **46-48**) gives a higher overall radiolabeling yield. From these results **47** has been chosen as a candidate for further antibody labeling studies. It was observed from control experiments that while the copper-bis-TSC complex appears to be stable over a short time-period, copper disassociates from the complex over a longer period of time (~ 1 day), which would discourage use of **47** for labeling of antibodies with radioisotopes of copper. However, substitution on the nitrogen in the 4-position of the thiosemicarbazone ligand has been shown to increase the ability of the bis-TSC ligand to bind copper more strongly against intracellular processes which allows for rapid blood clearance.[14] Future studies will also focus on the evaluation of a series of bis-thiosemicarbazones with different ^{4}N-alkyl group substitution to determine if this type of modification will enhance the ability of the ligand to bind strongly to copper. In addition we plan to synthesize a cyclic bis-TSC ligand and study the effect of this type of modification on the ability to bind copper under physiological conditions.

In conclusion, radiolabeling of antibodies with radioisotopes of copper can be performed under mild conditions utilizing bis-TSC chelates. This methodology avoids the harsh reducing conditions required for complexing radioisotopes such as technetium-99m to the antibody and thereby should help preserve the immunoreactivity of the antibody.

EXPERIMENTAL SECTION

General

All chemicals and solvents were analytical grade and were used without further purification unless otherwise noted. Melting points were obtained on a Thomas Hoover melting point apparatus and are reported uncorrected. The UV/visible spectra were obtained on a Varian Cary 219 spectrophotometer and the IR-spectra were obtained on a Beckman IR 4240 spectrophotometer. Nuclear magnetic resonance spectra were obtained using a Varian EM360A spectrometer using tetramethylsilane as an internal standard. Mass-spectra were determined by the Organic Spectroscopy Group, Analytical Chemistry Division at ORNL, by laser ionization FT mass spectrometry. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and all samples contained 0.25-1.5 mole ethanol after drying under vacuum (0.1 mm) at 80°C for 24 h. Copper-67 was produced by spallation either at Los Alamos National Laboratory or Brookhaven National Laboratory. The specific activity varied from 4.0 to 15 mCi/μg. Copper-64 was produced by copper-63 (n,γ) copper-64 reaction in the High Flux Brookhaven Reactor with a specific activity of 12 mCi/mg at the start of the synthesis. ITLC plates were purchased from Gelman Sciences (Ann Arbor, MI). RP-C18 Sep Pak were purchased from Waters Associates (Milford, Ma). Sephadex G-25-80, IgG, and BSA were purchased from Sigma Chemical Company.

p-Carboxymethyl-2-bromo-acetophenone (1)

Bromoacetyl chloride (3.43 g, 21.8 mmol) was added to a solution containing aluminum chloride (6.08 g, 45.6 mmol) in 50 mL methylene chloride. The solution was stirred for 15 min at room temperature and a solution of phenylacetic acid (3.12 g, 22.9 mmol) in 40 mL of methylene chloride was then slowly added. After addition was complete, the solution was refluxed for 1 h. The mixture was cooled to room temperature and poured into 150 mL of ice water. Methylene chloride (100 mL) was added and the mixture washed with 100 mL of a 6N HCl solution followed by washing twice with 100 mL of 1N HCl. The methylene chloride solution was dried over magnesium sulfate and evaporated to dryness to afford a pale yellow solid which was recrystallized from ether/petroleum ether to afford a white solid (3.84 g, 68%), mp, 103-105°C; ^{1}H NMR ($CDCl_3$) δ 8.1 (2H, d), 7.4 (2H, d), 4.5 (2H, s), 3.7 (2H, m). Compounds **2-6** were prepared in the same manner.

p-Carboxyethyl-2-bromo-acetophenone (2)

^{1}H NMR (acetone-d_6) δ 7.8 (2H, d), 7.2 (2H, d), 4.5 (2H, s), 2.6 (4H, m).

p-Carboxypropyl-2-bromo-acetophenone (3)

^{1}H NMR ($CDCl_3$) δ 8.0 (2H, d), 7.2 (2H, d), 4.4 (2H, s), 2.7 (2H, m), 2.4 (2H, m), 1.9 (2H, m).

p-Carboxybutyl-2-bromo-acetophenone (4)

^{1}H NMR ($CDCl_3$) δ 7.9 (2H, d), 7.3 (2H, d), 4.5 (2H, s), 2.8 (2H, m), 2.5 (2H, m), 1.8 (4H, m).

p-Carboxypentyl-2-bromo-acetophenone (5)

^{1}H NMR ($CDCl_3$) δ 8.0 (2H, d), 7.3 (2H, d), 4.5 (2H, s), 2.8 (2H, m), 2.5 (2H, m) 1.7 (6H, m).

p-Carboxyoctyl-2-bromo-acetophenone (6)

^{1}H NMR (acetone-d6) δ 8.0 (2H, d), 7.4 (2H, d) 4.7 (2H, s) 2.8-1.3 (16H, m).

p-Carboxymethyl-2-bromo-propiophenone (7)

2-Bromopropionyl chloride (4.42 g, 25.8 mmol) was added to a solution containing aluminum chloride (5.95 g, 44.6 mmol) in 50 mL of methylene chloride. After the solution had stirred for 15 min at room temperature, a solution of phenylacetic acid (3.56 g, 26.1 mmol) in 40 mL of methylene chloride was slowly added. After addition was complete, the reaction mixture was refluxed for 1 h and then allowed to cool to room temperature. The solution was poured into 200 mL of ice water and diluted with 100 mL of a 1N HCl solution. The mixture was washed with 100 mL 6N HC_2 solution and 200 ml water. The methylene chloride layer was dried over magnesium sulfate and evaporated to dryness to afford a yellow solid. The crude product was recrystallized from ether/petroleum ether to afford a pale yellow solid (1.84 g, 27%), mp, 144-146°C; ^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.3 (2H, d), 5.4 (1H, q), 3.7 (2H, s), 1.8 (3H, d). This procedure was also used preparation of compounds **8-13**.

p-Carboxyethyl-2-bromo-propiophenone (8)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.3 (2H, d), 5.4 (1H, q), 2.9 (4H, m), 1.8 (3H, d).

p-Carboxypropyl-2-bromo-propiophenone (9)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.3 (2H, d), 5.4 (1H, q), 2.8 (2H, m), 2.5 (2H, m), 1.9 (2H, m), 1.9 (3H, d).

p-Carboxybutyl-2-bromo-propiophenone (10)

^{1}H NMR ($CDCl_3$) δ 8.1 (2H, d), 7.3 (2H, d), 5.3 (1H, q), 2.8 (2H, m), 2.5 (2H, m), 2.0 (3H, d), 1.8 (4H, m).

p-Carboxypentyl-2-bromo-propiophenone (11)

^{1}H NMR ($CDCl_3$) δ 8.0 (2H, d), 7.2 (2H, d), 5.2 (1H, q), 2.7 (2H, m), 2.4 (2H, m), 1.9 (3H, d), 1.7 (6H, m).

p-Carboxyonyl-2-bromo-propiophenone (12)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.4 (2H, d), 5.7 (1H, q), 2.8-1.3 (18H, m), 1.9 (3H, d).

1-(4'-(p-(Carboxymethylphenyl)-phenyl)-2-bromo-propiophenone (13)

^{1}H NMR (acetone-d_6) δ 8.2-7.5 (8H, m), 5.7 (1H, q), 3.7 (2H, s), 1.9 (3H, d).

p-Carboxymethyl-1-nitrate-acetophenone (14)

A solution of compound 1 (2.75 g, 10.7 mmol) in 25 mL of acetonitrile was stirred at room temperature. A solution of silver nitrate (2.27 g, 13.4 mmol) in 25 mL of acetonitrile was slowly added and the solution allowed to stir for 24 h. The solution was then filtered to remove the silver bromide. The filtrate was diluted with 100 mL acetonitrile and washed three times with 100 mL of a saturated sodium chloride solution. The acetonitrile solution was then dried over magnesium sulfate and evaporated to dryness. The crude reaction mixture was washed in petroleum ether and filtered to afford the product as a pale white solid (2.20 g, 86%). The ^{1}H NMR (acetone-d_6) contained resonances at 8.1 (2H, d), 7.5 (2H, d), 6.0 (2H, s), 3.8 (2H, s). This compound was used without further purification and the above procedure was used in the preparation of compounds **15-26**.

p-Carboxyethyl-1-nitrate-acetophenone (15)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.5 (2H, d), 6.0 (2H, s), 3.1-2.6 (4H, m).

p-Carboxypropyl-1-nitrate-acetophenone (16)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.5 (2H, d), 6.0 (2H, s), 2.9 (2H, t), 2.5 (2H, t), 2.0 (2H, m).

p-Carboxybutyl-1-nitrate-acetophenone (17)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.5 (2H, d), 6.0 (2H, s), 2.8 (2H, m), 2.4 (2H, t), 1.7 (4H, m).

p-Carboxypentyl-1-nitrate-acetophenone (18)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.5 (2H, d), 6.1 (2H, s), 2.8 (2H, m), 2.3 (2H, t), 1.6 (6H, m).

p-Carboxyoctyl-1-nitrate-acetophenone (19)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.4 (2H, d), 6.0 (2H, s), 2.9-1.3 (16H, m).

p-Carboxymethyl-2-nitrate-propiophenone (20)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.4 (2H, d), 6.4 (1H, q), 3.7 (2H, s), 1.6 (3H, d).

p-Carboxyethyl-2-nitrate-propiophenone (21)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.6 (2H, d), 6.5 (1H, q), 3.0-2.3 (4H, m), 1.5 (3H, d).

p-Carboxypropyl-2-nitrate-propiophenone (22)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.4 (2H, d), 6.2 (1H, q), 2.8 (2H, t), 2.4 (2H, t), 2.1 (2H, m), 1.5 (3H, d).

p-Carboxybutyl-2-nitrate-propiophenone (23)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.4 (2H, d), 6.4 (1H, q), 2.8 (2H, m), 2.4 (2H, m), 2.1 (4H, m), 1.7 (3H, d).

p-Carboxypentyl-2-nitrate-propiophenone (24)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.4 (2H, d), 6.4 (1H, q), 2.8 (2H, m), 2.4 (2H, m), 2.1-1.0 (6H, m), 1.7 (3H, d).

p-Carboxynonyl-2-nitrate-propiophenone (25)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.4 (2H, d), 6.5 (1H, q), 2.8 (2H, t), 2.4 (2H, t), 1.7-1.0 (14H, m), 1.7 (3H, d).

1-(4'-(p-Carboxymethylphenyl)-phenyl)-2-nitrate-propiophenone (26)

^{1}H NMR (acetone-d_6) δ 8.2-7.3 (8H, m), 6.5 (1H, q), 3.6 (2H, s), 1.6 (3H, d).

p-Carboxymethylphenylglyoxal (27)

A solution of compound **14** (2.02 g, 8.4 mmol) in 8 mL of dimethylsulfoxide was cooled to 5°C. Sodium acetate trihydrate (136.1 mg, 1.0 mmol) was added and the solution stirred at 5°C for 6 h. The solution was poured into 200 mL of ice water and saturated with sodium chloride. The solution was then washed twice with 100 mL of ethyl acetate. The organic washes were combined and washed twice with a saturated sodium chloride solution and dried over magnesium sulfate. Evaporation of the solvent under vacuum afforded a yellow solid (1.09 g, 67%), ^{1}H NMR (acetone-d_6) δ 8.0 (2H, t), 7.4 (2H, d), 3.8 (1H, s). The compound was used without further purification. Compounds **28-32** were prepared in the same manner.

p-Carboxyethylphenylglyoxal (28)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, t), 7.5 (2H, m), 3.0-2.8 (4H, m).

p-Carboxypropylphenylglyoxal (29)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, t), 7.4 (2H, m), 2.8 (2H, t), 2.4 (2H, m), 2.0 (2H, m).

p-Carboxybutylphenylglyoxal (30)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, t), 7.4 (2H, m), 2.7 (2H, t), 2.4 (2H, m), 1.7 (4H, m).

p-Carboxypentylphenylglyoxal (31)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, t), 7.4 (2H, m), 2.8 (2H, m), 2.3 (2H, m), 1.6 (6H, m).

p-Carboxyoctylphenylglyoxal (32)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, t), 7.3 (2H, m), 2.2-1.3 (16H, m).

1-(p-Carboxymethylphenyl)-1,2-propandione (33)

To a solution of **20** (1.0 g, 4.0 mmol) in 10 mL of dimethylsulfoxide was added sodium acetate trihydrate (0.5 g, 3.9 mmol) and the mixture stirred at room temperature for 1 h. The solution was poured into 200 mL of ice water and the solution was saturated with sodium chloride. The solution was washed twice with 100 mL of ethyl acetate. The organic washes were combined and washed twice with a saturated sodium chloride solution and dried over magnesium sulfate. Evaporation of the solvent under vacuum afforded a yellow oil. The product was purified by Kugelruhr distillation and isolated as a yellow solid (0.57 g, 70%), mp, 101°C; ^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.5 (2H, d), 3.8 (2H, s), 2.5 (3H, s). Compounds **34-39** were prepared in the same manner.

1-(p-Carboxyethylphenyl)-1,2-propandione (34)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.5 (2H, d), 3.1 (2H, t), 2.7 (2H, t), 2.5 (3H, s).

1-(p-Carboxypropylphenyl)-1,2-propandione (35)

^{1}H NMR (acetone-d_6) δ 8.0 (2H, d), 7.5 (2H, d), 2.8 (2H, m), 2.4 (2H, m), 2.5 (3H, s), 2.0 (2H, m).

1-(p-Carboxybutylphenyl)-1,2-propandione (36)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.5 (2H, d), 3.1 (2H, m), 2.7 (2H, m), 2.5 (3H, s), 1.8 (4H, m).

1-(p-Carboxypentylphenyl)-1,2-propandione (37)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.5 (2H, d), 3.1 (2H, m), 2.7 (2H, m), 2.5 (3H, s), 1.8 (6H, m).

1-(p-Carboxynonylphenyl)-1,2-propandione (38)

^{1}H NMR (acetone-d_6) δ 8.1 (2H, d), 7.5 (2H, d), 2.8 (2H, m), 2.5 (3H, s), 2.4 (2H, m), 1.8-1.2 (14H, m).

1-(4'-(p-Carboxymethylphenyl)-phenyl)-1,2-propandione (39)

^{1}H NMR (acetone-d_6) δ 8.2-7.0 (8H, m), 3.7 (2H, s), 2.5 (3H, s).

p-Carboxymethylphenylglyoxal bis 4-methyl-3-thiosemicarbazone (40)

A solution of 4-methyl-3-thiosemicarbazide (1.52 g, 14.5 mmol) in 50 mL of a 1N HCl solution was heated to 60°C. Compound **27** (1.09 g, 5.7 mmol) in 5 mL of ethanol and 5 mL of water was then added dropwise to the thiosemicarbazide solution. After addition was complete the

solution was refluxed for 2 h, slowly cooled to room temperature and then placed in the freezer overnight. The solution was filtered and the product recrystallized twice from ethanol/water to afford an orange yellow solid (0.76 g, 36%), mp, 202-204°C; ^{1}H NMR (DMSO-d_6) δ 12.3 (1H, s), 11.8 (1H, s), 8.9 (1H, bs), 8.3 (1H, s), 7.7 (2H, d), 7.4 (2H, d), 3.7 (2H, s), 3.1 (6H, m); laser FT mass spectrum m/z 365 (M-1); IR (KBr) 1725 cm^{-1}. Anal ($C_{14}H_{18}S_2N_6O_2$) C,H,N,S. Compounds **41-52** were prepared in the same manner.

p-Carboxyethylphenylglyoxal bis 4-methyl-3-thiosemicarbazone (41)

^{1}H NMR (DMSO-d_6) δ 12.3 (1H, s), 11.8 (1H, s), 8.9 (1H, bs), 8.2 (1H, s), 7.8 (2H, d), 7.4 (2H, d), 3.1 (6H, s), 2.7 (4H, m); laser FT mass spectrum m/z 379 (M-1); IR (KBr) 1745 cm^{-1}.

p-Carboxypropylphenylglyoxal bis 4-methyl-3-thiosemicarbazone (42)

^{1}H NMR (DMSO-d_6) δ 12.3 (1H, s), 11.8 (1H, s), 8.9 (1H, bs), 8.2 (1H, s), 7.8 (2H, d), 7.3 (2H, d), 3.1 (6H, s), 2.7 (2H, m), 2.3 (2H, m), 1.9 (2H, m); laser FT mass spectrum m/z 393 (M-1); IR (KBr) 1708 cm^{-1}.

p-Carboxybutylphenylglyoxal bis 4-methyl-3-thiosemicarbazone (43)

^{1}H NMR (DMSO-d_6) δ 12.2 (1H, s), 11.8 (1H, s), 8.8 (1H, bs), 8.2 (1H, s), 7.7 (2H, d), 7.3 (2H, d), 3.1 (6H, s), 2.6 (2H, m), 2.3 (2H, m), 1.6 (4H, m); laser FT mass spectrum m/z 407 (M-1); IR (KBr) 1720 cm^{-1}.

p-Carboxypentylphenylglyoxal bis 4-methyl-3-thiosemicarbazone (44)

^{1}H NMR (DMSO-d_6) δ 12.3 (1H, s), 11.7 (1H, s), 8.8 (1H, bs), 8.2 (1H, s), 7.8 (2H, d), 7.3 (2H, d), 3.1 (6H, s), 2.6 (2H, m), 2.2 (2H, m), 1.4 (6H, m); laser FT mass spectrum m/z 421 (M-1); IR (KBr) 1730 cm^{-1}.

p-Carboxyoctylphenylglyoxal bis-4-methyl-3-thiosemicarbazone (45)

^{1}H NMR (acetone-d_6) δ 13.6 (1H, s), 8.7 (1H, s), 7.7 (2H, d), 7.3 (2H, d), 3.1 (6H, s), 2.7-1.2 (16H, m); IR (neat) 1740, 1710 cm^{-1}.

1-(p-Carboxymethylphenyl)-1,2-propandione bis-4-methyl-3-thiosemicarbazone (46)

^{1}H NMR (DMSO-d_6) δ 11.7 (1H, s), 8.7 (1H, bs), 7.5 (2H, d), 7.2 (2H, d), 3.8 (2H, s), 3.1 (3H, d), 2.9 (3H, d), 2.5 (3H, s); laser FT mass spectrum m/z 379 (M-1); IR (KBr) 1730 cm^{-1}.

1-(p-Carboxyethylphenyl)-1,2-propandione bis 4-methyl-3-thiosemicarbazone (47)

^{1}H NMR (DMSO-d_6) δ 10.8 (1H, s), 8.8 (1H, s), 7.4 (2H, d), 7.2 (2H, d), 3.1 (3H, d), 2.9 (3H, d), 2.5 (4H, m), 2.5 (3H, s); laser FT mass spectrum m/z 393 (M-1); IR (KBr) 1740, 1710 cm^{-1}.

1-(p-Carboxypropylphenyl)-1,2-propandione bis 4-methyl-3-thiosemicarbazone (**48**)

^{1}H NMR (DMSO-d_6) δ 10.4 (1H, s), 8.7 (1H, s), 7.4 (2H, d), 7.2 (2H, d), 3.1 (3H, d), 2.9 (3H, d), 2.7 (2H, m), 2.5 (3H, m), 2.3 (2H, s), 1.9 (2H, m); laser FT mass spectrum m/z 407 (M-1); IR (KBr) 1740, 1710 cm^{-1}.

1-(p-Carboxybutylphenyl)-1,2-propandione bis 4-methyl-3-thiosemicarbazone (**49**)

^{1}H NMR (DMSO-d_6) δ 10.3 (1H, s), 8.7 (1H, s), 7.4 (2H, d), 7.2 (2H, d), 3.1 (3H, d), 2.9 (3H, d), 2.8 (2H, m), 2.5 (3H, s), 2.3 (2H, m), 1.7 (4H, m); laser FT mass spectrum m/z 421 (M-1); IR (KBr) 1710, 1680 cm^{-1}.

1-(p-Carboxypentylphenyl)-1,2-propandione bis 4-methyl-3-thiosemicarbazone (**50**)

^{1}H NMR (acetone-d_6) δ 9.5 (1H, s), 8.6 (1H, s), 7.3 (2H, d), 7.1 (2H, d), 3.1 (3H, d), 2.9 (3H, d), 2.7 (2H, m), 2.5 (3H, s), 2.3 (2H, m), 1.6 (6H, m); IR (KBr) 1740, 1720 cm^{-1}.

1-(p-Carboxynonylphenyl)-1,2-propandione bis 4-methyl-3-thiosemicarbazone (**51**)

^{1}H NMR (acetone-d_6) δ 9.6 (1H, s), 8.6 (1H, s), 7.4 (2H, d), 7.1 (2H, d), 3.1 (3H, d), 2.9 (3H, d), 2.6 (2H, m), 2.5 (3H,s), 2.3 (2H, m), 1.3 (14H, m); laser FT mass spectrum m/z 491 (M-1); IR (neat) 1740, 1710 cm^{-1}.

1-(4'-(p-Carboxymethylphenyl)-phenyl)-1,2-propandione bis 4-methyl-3-thiosemicarbazone (**52**)

^{1}H NMR (DMSO-d_6) δ 10.7 (1H, s), 9.0-8.7 (2H, m), 8.0-7.1 (9H, m), 7.1 (2H, d), 3.6 (2H, s), 3.1 (3H, d), 2.8 (3H, d), 2.4 (3H, s); laser FT mass spectrum m/z 455 (M-1); IR (KBr) 1730 cm^{-1}.

Copper-labeled p-carboxypentylphenylglyoxal bis 4-methyl-3-thiosemicarbazone tetrafluorophenol ester (**53**)

Cupric acetate (1.0 mg, 7.4 μmol) and **44** (2.9 mg, 6.9 μmol) were dissolved in 20 μL of an ethanol:DMSO (1:1) solution. To this solution was added 100 μL of a 0.2M acetate buffer (pH 3.1) and the mixture was heated at 70°C for 15 min. The solution was then diluted with 2 mL of water and loaded onto a RP-C18 Sep Pak. The Sep Pak was washed with 3x3 mL water and the copper complex eluted with 2.0 mL of acetonitrile; TLC (RP-C18, acetonitrile:water (4:1)) R_f=0.60. The solution was evaporated to dryness under a stream of nitrogen to afford a red residue. A mixture of 100 μL of a 0.2M sodium phosphate solution (pH 6.0), 100 μL of a tetrafluorophenol solution (100 mg/mL of 90% acetonitrile) and 100 μL in a 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide solution (125 mg/mL in 90% acetonitrile) was added to the residue. The solution was stirred at 70°C for 30 min and cooled to room temperature. The solution was diluted to 2 mL with water and loaded onto a RP-C18 Sep Pak. The Sep Pak was washed with 3x3 mL of water and the product was eluted with 2 mL of acetonitrile; TLC (RP-C18, acetonitrile:water (4:1)) R_f=0.38. TLC analysis indicated the reaction went to completion with no starting material present.

Preparation of IgG-47 complex

Compound **47** (19.0 mg, 48.2 μmol) was dissolved in dry DMF (0.5 ml) and cooled to 0°C in an ice salt bath. To the solution was added triethylamine (5.1 mg, 50.2 μmol) and diphenylphosphoryl azide (14.0 mg, 51.0 μmol). The mixture was stirred at 0°C for one hour. An aliquot of the ligand solution (20 μl) was then added slowly to a vial containing IgG (21.5 mg) in 0.05 M borate buffer (pH 9.5) (1 ml) at 0°C. The protein ligand solution was stirred at 0°C for 2 hours and purified by passage through a Sephadex G-25 column. The fraction containing protein-ligand complex was dialyzed against water for 24 hours in the refrigerator followed by lyophilization to afford the protein-ligand complex as a white solid. The protein to ligand ratio was determined to be 1.0 by UV analysis at 340 nm and 280 nm for **47** and 280 nm for IgG[8].

Preparation of Copper Complex for UV/visible analysis

Each ligand (1×10^{-5} mol) was dissolved in 10 mL of ethanol. One mL of a 1×10^{-3} M copper acetate solution in 0.2 M acetate buffer (pH 3.1) was added to one mL of the ligand solution. The solution was kept a room temperature for 15 min and 1 mL of the solution was diluted to 10 mL with ethanol and analyzed by UV/visible spectroscopy.

Labeling BSA and IgG with Copper-64 or Copper-67

To a solution of ligand (0.5 μmol) in 200 μL of ethanol was added 100 μL of 0.2 M acetate buffer (pH 3.1) and a solution of copper-64 or copper-67 chloride in aqueous ethanol. The solution was stirred at 70°C for 15 min and cooled to room temperature. The solution was then diluted to 10 mL with water and loaded onto a RP-C18 Sep Pak which was washed with 3x3 mL of water followed by 0.25 mL of acetonitrile. The product was eluted from the Sep Pak with 2.0 mL of acetonitrile as a red-colored solution. No free copper was observed by TLC analysis (RP-C18, acetonitrile/water (4:1)). The product solution was evaporated to dryness under a stream of argon and 100 μL of a 0.2 M phosphate buffer solution (pH 6.0), 100 μL of a tetrafluorophenol/90% acetonitrile solution (100 mg/mL) and 100 μL of a 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide/90% acetonitrile solution (125 mg/mL) then added to the residue. The solution was stirred at 70°C for 30 min and cooled to room temperature. TLC analysis indicated that esterification proceeded in > 70% as compared to cold standard. The solution was diluted with 10 mL of water and loaded onto a RP-C18 Sep Pak and washed with 3x3 mL water followed by 0.25 mL of acetonitrile. The product was eluted from the Sep Pak with 2.0 mL acetonitrile and evaporated to dryness under a stream of argon. The residue was then dissolved in 100 μL of DMSO and followed by the addition of 500 μL of a 0.2 M sodium phosphate buffer (pH 9.0) and 500 μL of a solution of BSA in saline (2.5 mg/mL). After the solution was gently stirred at room temperature for 15 min 250 μL of lysine (100 mg/mL) was added. For IgG 1 mg of the protein was dissolved in 1 ml of a 0.2 M sodium phosphate buffer (pH 9.0) and added to the vial containing the copper-DMSO solution. The solution was then stirred at 37 °C for 30 minutes. The solution was purified by passage through a Sephadex G-25 column using a 0.2 M phosphate buffer (pH 9.0), and the product was collected in the solvent front.

Labeling of IgG-47 with copper-67

IgG-47 complex (1 mg) was dissolved in a 0.1 M acetate buffer (pH 6) and then an aliquot of the copper-67 chloride solution was added. The solution was stirred at 37 °C for 30 minutes and then purified by passage through a Sephadex G-25 column. The product was collected in the solvent front and analyzed by ITLC using 0.1 M $NaHCO_3$ as the mobile phase.

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