BLEDTA II: SYNTHESIS OF A NEW TUMOR-VISUALIZING DERIVATIVE OF CO(III)-BLEOMYCIN

Leslie H. DeRiemer, Claude F. Meares
Department of Chemistry, University of California, Davis, CA 95616.

David A. Goodwin and Carol I. Diamanti
Stanford University School of Medicine and Veterans Administration
Medical Center, Palo Alto, CA 94304.

SUMMARY

A new bifunctional chelating agent, (S)-1-(p-bromoacetamidobenzyl) ethylenedinitrilotetraacetic acid, has been prepared from L-phenylalanine. Reaction of the chelating agent with the Co(III) complex of the antitumor antibiotic bleomycin-A_2DM was used to generate a new derivative of Co(III)-bleomycin, BLEDTA II. The product can be radiolabeled quickly by binding a radioactive metal ion to the EDTA group. The in vivo distribution of ^{111}In-BLEDTA II in tumor-bearing mice was determined and found to be indistinguishable from that of the clinically useful ^{111}In(III)-BLEDTA I complex (J. Med. Chem. 22: 1019 (1979)).

Key Words: Bifunctional Chelating Agents, Cobalt, Bleomycin, Indium, Tumor Visualization

INTRODUCTION

The antitumor antibiotic bleomycin has been shown to selectively concentrate within the cells of some cancers (1, 2). Recently we described the preparation of a clinically useful radiopharmaceutical, "BLEDTA", which also localizes in tumors (3). BLEDTA (now denoted BLEDTA I) was made by coupling the terminal amine of Co(III)-bleomycin-A_2DM with an ethylenedinitrilotetraacetic acid (EDTA) derivative. Because the EDTA group is a powerful metal-chelating agent which is able to form stable complexes with a number of metal ions (4), BLEDTA I can be radiolabeled immediately before use by combining it with a solution containing a radioactive metal ion. In vivo work with a tumor-bearing mouse model system and with human cancer patients has shown that the ^{111}In(III)-BLEDTA I chelate accumulates in

0362-4803/81/101517-18$01.80

Received October 28, 1980
Revised December 23, 1980

neoplastic tissue and is a useful diagnostic tool for determining both the size and location of malignancies in cancer patients (4, 5).

This paper describes the synthesis and in vivo characteristics of a related bleomycin analog, BLEDTA II, which is prepared as outlined in Figure 1. The chelating portion of BLEDTA II is much easier to prepare than the related EDTA derivative used to make BLEDTA I. The "bifunctional chelating agent" used to synthesize BLEDTA II, (S)-1-(p-bromoacetamidobenzyl)ethylenedinitrilotetraacetic acid, was prepared from L-phenylalanine.

The synthesis of chelating agents from amino acids was recently described by Yeh et al. (6) and offers several advantages over the previous method of preparing chelating agents (13). The new synthetic route is simpler, proceeds with retention of stereochemistry about the asymmetric carbon and can originate from an interesting variety of available starting materials. In human serum the ^{111}In(III) complexes of chelating agents prepared from amino acids exhibit the same low rates of metal ion loss shown by other EDTA derivatives containing a substituent on an ethylene carbon of EDTA (7).

MATERIALS AND METHODS

Chemical Procedures. Reagents.

L-phenylalanine was purchased from Sigma. Blenoxane (bleomycin sulfate) was generously provided by Dr. W. T. Bradner of Bristol Laboratories. Carrier free $^{111}InCl_3$ (in 0.05 N HCl, 0.9% NaCl) was purchased from Medi-Physics, Emeryville, CA, and was purified as described previously (8). Columns were monitored either at 254 nm (1-(p-nitrobenzyl) EDTA preparation) or at 280 nm (bleomycin and bleomycin derivatives). AG-50 and AG-1 ion exchange resins were purchased from Bio-Rad, and Sephadex C-25 and A-25 ion exchange resins were from Pharmacia; linear gradients were used throughout. Borane-tetrahydrofuran complex was from Aldrich. High-pressure liquid chromatography

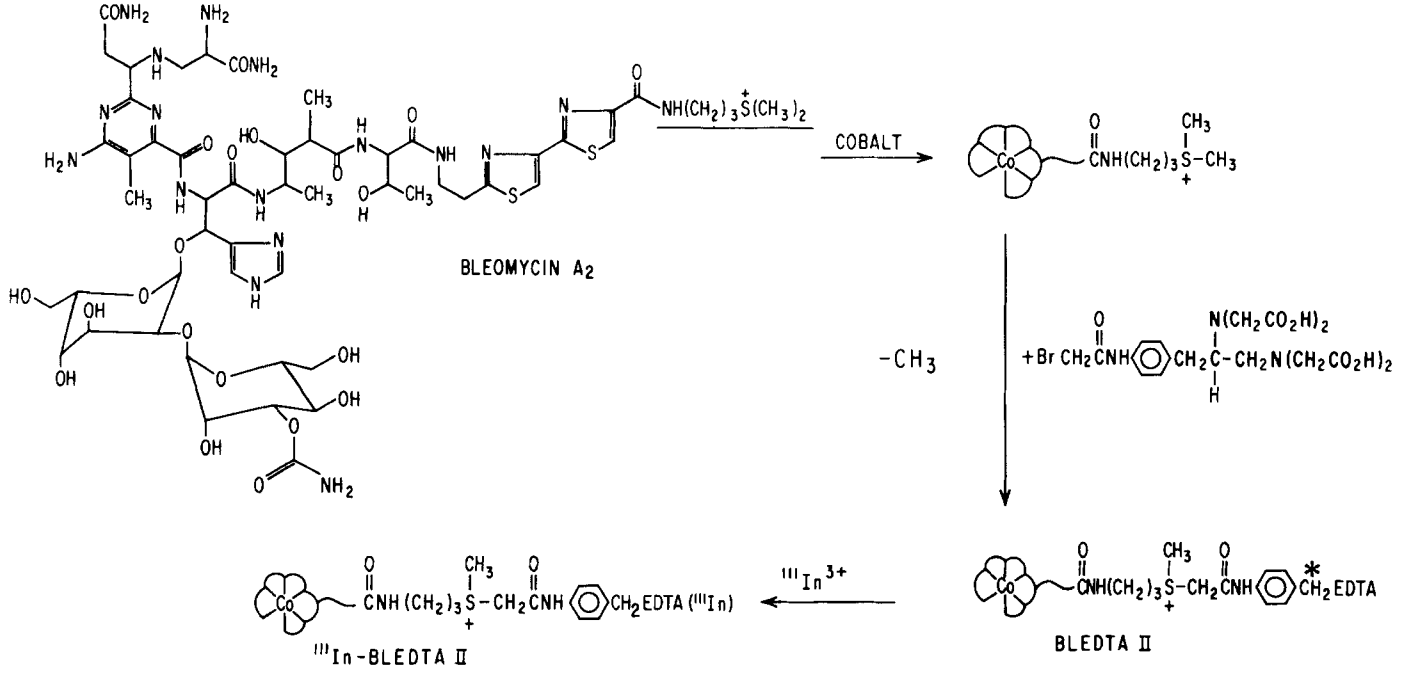

Figure 1. Synthesis of BLEDTA II. Bleomycin A_2 is shown in detail; the terminal amine is underlined. After the Co(III)-bleomycin A_2 complex (shown schematically with only the terminal amine portion shown in detail) is isolated, it is demethylated to Co(III)-bleomycin A_2DM, then alkylated with 1-(p-bromoacetamidobenzyl) EDTA to give BLEDTA II. BLEDTA II contains half the stereoisomers and one more methylene group (indicated with an asterisk) than BLEDTA I.

was run on a Waters 3.9 mm x 30 cm C_{18} μ-bondapak column with 1% linear aqueous ammonium acetate/methanol (6:4, v:v) as the solvent. Thin-layer chromatography (TLC) was run on Merck silica gel 60 or 60F plates developed in solvent 1, (95% ethanol:6.5 M NH_4OH, 4:1 v:v) or solvent 2 (10% (w/v) aqueous NH_4OAC:methanol, 1:1, v:v). Doubly distilled water was used throughout and labware was acid washed to avoid heavy metal contamination (9). Elemental analyses were performed by Galbraith Analytical Labs, Inc., and were within ± 0.4% of the theoretical value. FT ^1H NMR (360 MHz) spectra were taken by Dr. Richard R. Anderson using a Nicolet instrument at the UCD Magnetic Resonance Facility. Chemical shifts were reported relative to HDO (δ 4.8 ppm). CH_3CH_2SH was purchased from Eastman and was distilled before use.

Co(III)-Bleomycin A_2

The original procedure for preparation of Co(III)-bleomycins (3) was modified so that most of the Co(III)-bleomycin could be isolated as the thermodynamically stable orange Co(III)-bleomycin isomer.

The contents of 19 vials of the commercially available mixture of bleomycin (Blenoxane) were dissolved in 10 ml of H_2O; the concentration of bleomycin was determined by reading the absorbance of the solution at 292 nm, using a molar extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} (10). This solution, which contained 105 μmol of bleomycin, was diluted to 22 ml and 1.1 ml of a 0.10 M $CoCl_2$ solution was added (ratio of Co(II)/bleomycin = 1.05). By adding approximately 30 μl of 6 M NaOH, the pH of the solution was adjusted to 7.0. O_2 was bubbled through the solution for 3 min; the 60 ml nalgene bottle containing the solution was then sealed and left in a 50°C bath for 18 h.

The dark green-brown solution and 5 ml of H_2O (rinse) were transferred to a 100 ml roundbottom flask with a reflux condenser. The solution was

heated at 110°C for 3 h. The resulting orange solution was applied to a 1 x 70 cm Sephadex C-25 column (NH_4^+ form) and eluted with a 1 ℓ gradient of 0.1 M to 0.5 M ammonium formate, pH 6.5. The flow rate of the column was 0.9 ml/min; 8 ml fractions were collected. The column was run at 4°C. Figure 2A shows the absorbance at 452 nm of every third fraction. The yield of pure orange Co(III)-bleomycin A_2 was determined by reading the absorbance of a solution at 452 nm and using a molar extinction coefficient of 214 M^{-1} cm^{-1} (3); this indicated 44 μmol of orange Co(III)-bleomycin A_2 (42% yield).

Co(III)-Bleomycin A_2DM

Co(III)-bleomycin A_2DM was prepared from Co(III)-bleomycin A_2 by reaction with a mercaptide using our published procedure (3). Ethyl mercaptide was used instead of methyl mercaptide because it was easier to handle; presumably, other mercaptides could also be used. Starting with 30.8 μmol of Co(III)-bleomycin A_2, the yield was 21.7 μmol (70%) of Co(III)-bleomycin A_2DM.

(S)-p-Nitrophenylalanine

p-Nitrophenylalanine was prepared by the Beilstein procedure (11). 10.0 g (60.6 mmol) of L-phenylalanine was dissolved in 16 ml of concentrated H_2SO_4 (95-98%) at 0°C. The temperature of the solution was maintained at 0°C as 3.0 ml of 90% HNO_3 was added dropwise with magnetic stirring. After standing at 0°C for 10-15 min following the addition of HNO_3, the solution was poured over 200 ml of ice and subsequently diluted to 700 ml with H_2O. The solution was then heated to boiling and neutralized with $Pb(CO_3)_2$ (80 g, 0.24 mmol). This mixture was filtered and the supernatant treated with H_2S(g) to precipitate Pb^{2+}. After a second filtration the solution was reduced to 1/3 its volume by rotary evaporation. p-nitrophenylalanine precipitated and was collected by filtration and washed with 95% ethanol.

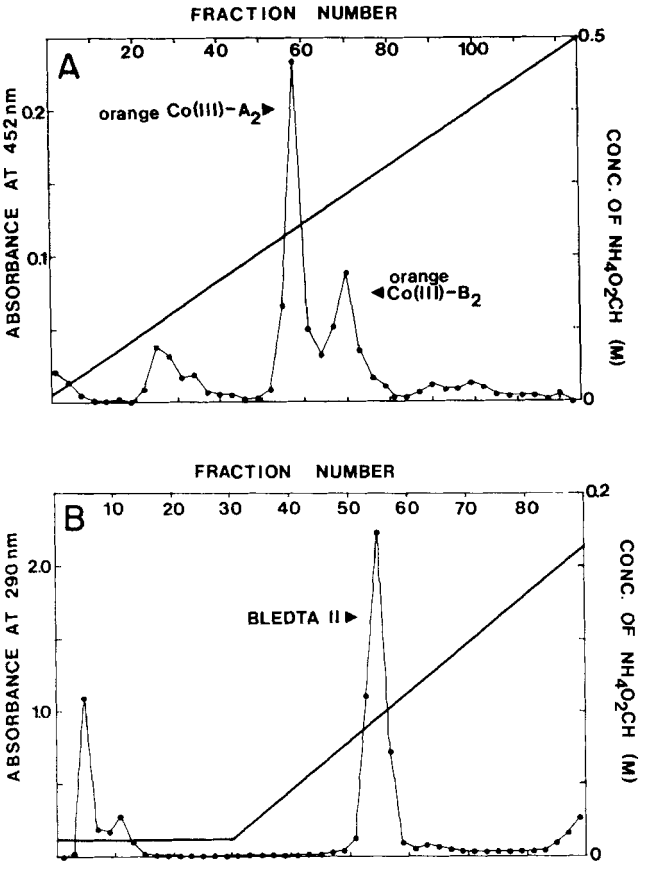

Figure 2. Column Elution Profiles.

a. Isolation of orange Co(III)-bleomycin A_2 by elution from a 1 x 70 cm Sephadex C-25 column, NH_4^+ form. A gradient of 0.1 M to 0.5 M NH_4O_2CH, pH 6.5, was used (diagonal line). The peaks corresponding to Co(III)-bleomycin A_2 and B_2 are shown. The column was run at 4°C.

b. Purification of BLEDTA II by elution from a 1 x 45 cm Sephadex A-25 column, acetate form. A wash with 0.01 M NH_4O_2CH, pH 8, was followed by 500 ml gradient of 0.01 M to 0.3 M NH_4O_2CH, pH 8 (diagonal line). The column was run at 4°C.

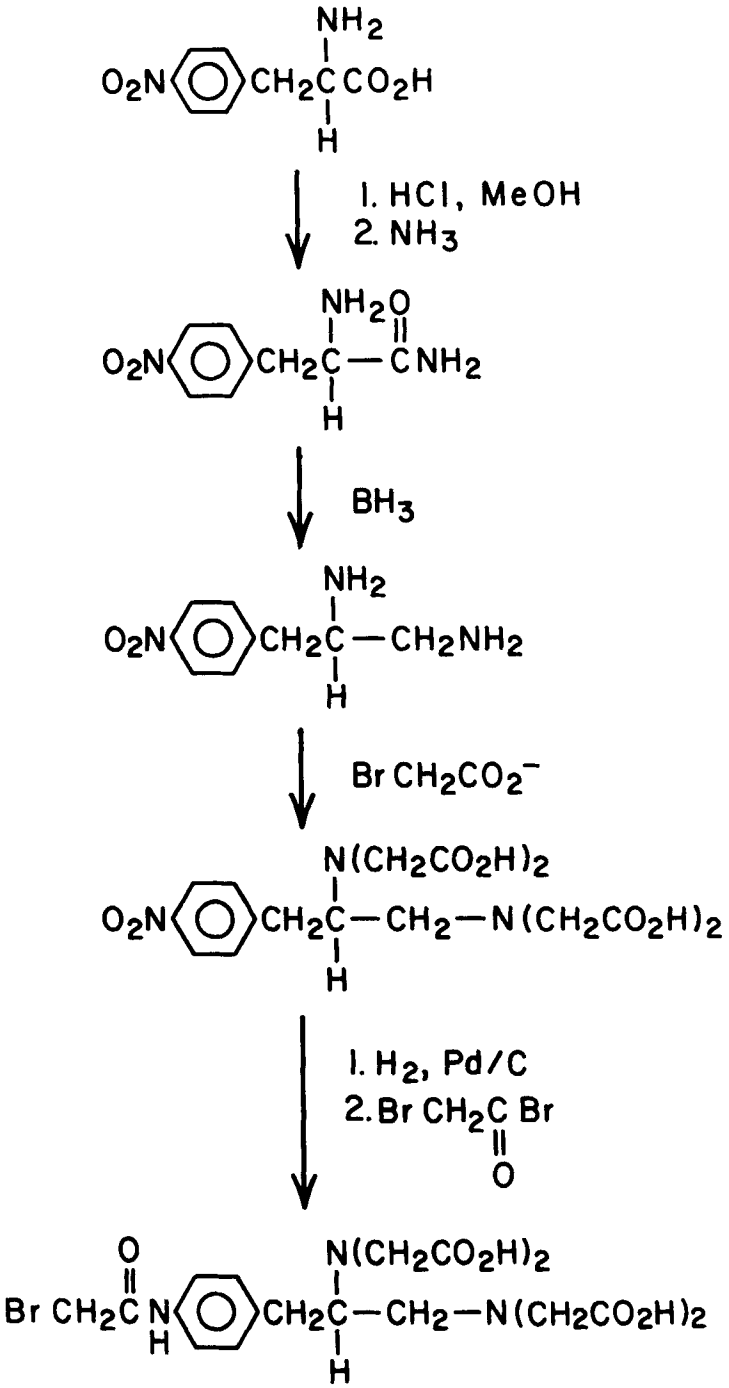

Figure 3. Synthesis of 1-(p-bromoacetamidobenzyl) EDTA from p-nitrophenylalanine. The overall yield was 8.7%.

Following recrystallization from boiling H_2O, the yield was 6.36 g (55%). Using solvent 1, the product had an R_f of 0.58.

(S)-p-Nitrophenylalanine methyl ester

The methyl ester of p-nitrophenylalanine was prepared by reaction in HCl-saturated methanol as previously described (6). Using solvent 1, the product had an R_f of 0.78. Yield was 90-95%.

(S)-p-Nitrophenylalanine amide

The amide was prepared from the ester hydrochloride by reaction in NH_3-saturated methanol (12). After taking the reaction mixture to dryness by rotary evaporation, p-nitrophenylalanine amide hydrochloride was recrystallized from absolute ethanol. Yield was 3.5 g (90%). The product melted at 235°C and had an R_f of 0.78 with solvent 1, and an R_f of 0.70 with solvent 2.

(S)-1-(p-Nitrobenzyl)ethylenediamine

1.50 g of p-nitrophenylalanine amide hydrochloride was dissolved in 75 ml of THF and reduced with BH_3•THF (6). The reaction mixture was taken to dryness, taken up in H_2O and purified by chromatography on an AG-50 column (H^+ form, 0.7 x 18 cm) with a 0 to 7 M HCl gradient. The product was eluted with 6-7 M HCl and was lyophilized. The diamine dihydrochloride had an R_f of 0.50 in solvent 2. Yield was 1.0 g (70%) Anal. ($C_9H_{15}Cl_2N_3O_2$), C, Cl, H, N.

(S)-(p-Nitrobenzyl) EDTA

A sample of 0.40 g of the diamine dihydrochloride was carboxymethylated with bromoacetic acid in alkaline solution as described (6). Yield was 531 mg (75%). On TLC with solvent 2, the R_f of the product was 0.78. Anal. ($C_{17}H_{21}N_3O_{10}$•5/2 H_2O) C, H, N.

(S)-(p-Bromoacetamidobenzyl) EDTA

(S)-p-(Aminobenzyl) EDTA was prepared by reduction of (S)-(p-nitrobenzyl) EDTA as reported earlier (3). 120 mg (303.5 μmol) of (p-aminobenzyl) EDTA was dissolved in 500 μl of H_2O and the bromoacetamido compound prepared as described for 1-(p-bromoacetamidophenyl) EDTA (3). Yield was 32.07 mg (61.8 μmol; 20.4%) of precipitated (p-bromoacetamidobenzyl) EDTA. The mother liquor was not worked for a second crop. TLC with solvent 2 showed one spot with an R_f of 0.96; this spot was 4-(p-nitrobenzyl) pyridine positive (20) and quenched fluorescence.

BLEDTA-II

The conditions for the coupling reaction were similar to those described for BLEDTA I (3). 6.18 μmol of Co(III)-bleomycin A_2DM and 61.8 μmol of (S)-(p-bromoacetamidobenzyl) EDTA were combined with aqueous solution with a final volume of 1.87 ml. The pH of the solution was adjusted to 4.4 by the addition of 20 μl of 6 M NaOH and 5 μl of 12 M HCl. The reaction was allowed to proceed at room temperature and was monitored by HPLC. With the HPLC system used, product and free chelate were eluted immediately, while Co(III)-bleomycin A_2DM was retained slightly. After 6 h the pH of the reaction mixture was adjusted to 8.2 and the mixture was applied to a Sephadex A-25 column. The column (1 x 45 cm) was first washed with 150 ml of 0.1 M NH_4O_2CH, pH 8, followed by a 500 ml gradient of 0.01 M to 0.3 M NH_4O_2CH pH 8. The column wash and gradient were done at 4°C. The absorbance at 452 nm of every other fraction was determined. The product was eluted with 0.08 M NH_4C_2CH, as shown in Figure 2B. Fractions containing product were pooled and lyophilized for two days to remove solvent and excess salt. TLC with solvent 1 of the product gave one spot with an R_f of 0.46; autoradiography of a TLC plate of the ^{111}In(III) chelate of the product showed only one spot with an R_f of 0.4.

As shown in Figure 4, the 360 MHz proton NMR spectrum of the product contained an ab pattern centered at 7.15 ppm, corresponding to the aromatic protons on the benzyl-EDTA moiety. The position of the terminal amine methyl proton resonance (3.1 ppm) confirmed that the terminal amine thioether had been alkylated to give a sulfonium ion.

Yield of the product, BLEDTA II, was determined to be 2.83 μmol (46%) by reading the absorbance of a solution at 452 nm and using a molar extinction coefficient of 214 M^{-1} cm^{-1} (3).

Preparation of ^{111}In(III)-BLEDTA II

50 μl of 1-2 mCi of $^{111}InCl_3$ in 0.01 M HCl was added to an equal volume of 0.5 mM BLEDTA II. After vortexing, 50 μl of 0.1 M $NaHCO_3$ was added to neutralize the solution. Prior to injection, the solution was diluted with saline and sterilized by filtration as previously described (3).

Distribution in Tumor-Bearing Mice

After injection of each radiolabeled compound into the tail veins of specially prepared BALB/c mice, the organ distribution and tumor uptake of radioactivity were determined by methods described elsewhere (13).

RESULTS AND DISCUSSION

Chemistry

Figure 3 summarizes the preparation of the bifunctional chelating agent 1-(p-bromoacetamidobenzyl) EDTA. The configuration about the asymmetric carbon is retained throughout the synthesis; this is important because stereochemically pure compounds are valuable as probes of biological systems. As described by Yeh et al. (6) substitution of a primary amine for NH_3 in the preparation of the amide is an effective method for synthesizing many

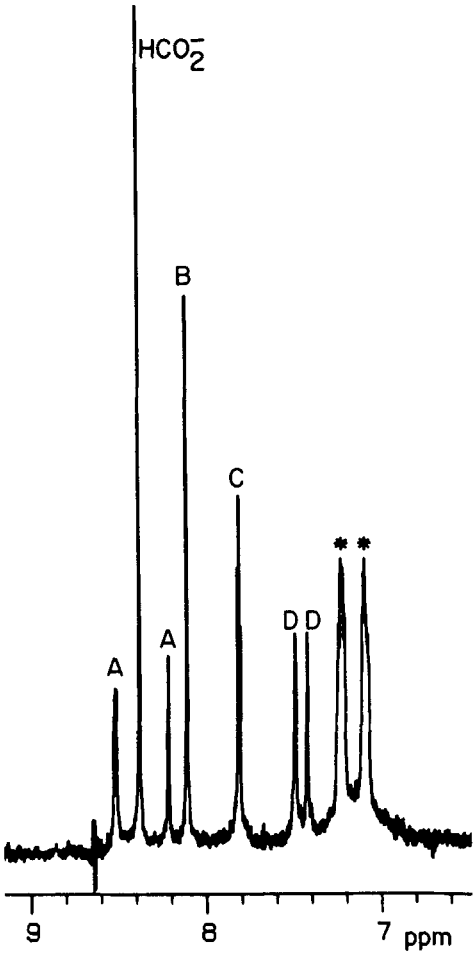

Figure 4. Aromatic region of the 360 MHz proton NMR spectrum of BLEDTA II, taken at a pH meter reading of 4.9. The peak due to formate ion (from the last column chromatography) is indicated. Resonances (A) and (D) are assigned to the protons on C_2 and C_4, respectively, of the β-hydroxyhistidine residue. Peak (B) is assigned to the proton on the thiazole ring nearest the terminal amine. Peak (C) is assigned to the proton on the thiazole ring adjacent to the L-threonine residue. The resonances of the p-substituted benzene ring of the chelating agent are indicated with asterisks. The resonances are assigned in accord with NMR studies of bleomycins (19) and metallobleomycins (22-24).

different chelating agents from a single amino acid.

The synthesis of BLEDTA II is outlined in Figure 1. The structure of bleomycin A_2 is shown in detail; the terminal amine moiety is underlined. Coordination of Co(III) in the intrinsic metal-binding site of bleomycin protects the β-aminoalanine and β-hydroxyhistidine residues from reaction with the alkylating bifunctional chelating agent 1-(p-bromoacetamidobenzyl) EDTA, and also prevents non-specific binding of the metal ion during the radiolabeling step. Because of its d^6 electronic configuration, the Co(III) ion is inert to ligand exchange (14) and is not transferred to the EDTA moiety of the final product, BLEDTA II. Two different Co(III) bleomycin complexes have been described (3, 15, 16); the thermodynamically more stable orange Co(III) complex was used throughout the synthesis of BLEDTA II.

The Co(III)-bleomycin A_2 complex was isolated by cation-exchange chromatography, as Figure 2A shows, and demethylated by reaction of the terminal amine sulfonium ion with a mercaptide. Side reactions were avoided by preparing the mercaptide solution immediately before the demethylation reaction; mercaptide solutions that had been stored desiccated for only a few days at -70°C were found to give undesirable products. Solid sodium ethylmercaptide, which had been stored desiccated at 4°C up to 2 days, was still reactive without generating side products.

The β-hydroxyhistidine and pyrimidine residues are coordinated to the Co(III) ion; the proton resonances corresponding to them are shifted downfield upon formation of orange Co(III) bleomycin, and the histidine imidazole resonances (pk_a = 5.0 (21)) cannot be titrated between a pH meter reading of 3.1 and 7.9 (unpublished data).

Demethylation of Co(III)-bleomycin A_2 is accompanied by a splitting of the histidine imidazole and pyrimidine methyl proton resonances (Figure 4 and ref. (3)), which suggests that orange Co(III)-bleomycin A_2DM is a mixture of compounds differing in the coordination of ligands about Co(III). As

discussed previously, the mercaptide is not coordinated to Co(III) (3). Due to the basicity of the mercaptide, the possibility that epimerization of isomerization of bleomycin has occurred cannot be entirely eliminated. Another possibility would be displacement of an oxygen-containing ligand (e.g. the carbamoyl carboxyl group) by OH^- generated when the mercaptide reaction mixture is applied to the cation exchange column. In vivo studies in mice with orange ^{57}Co-bleomycin A_2DM have shown that its organ distribution is indistinguishable from that of orange ^{57}Co-bleomycin A_2. Therefore, the structural change which is manifested in the NMR spectrum does not appear to affect the biological transport properties of the molecule.

The final step in the preparation of BLEDTA II is alkylation of Co(III)-bleomycin A_2DM with (S)-1-(p-bromoacetamidobenzyl) EDTA. The elution profile of the Sephadex A-25 column is shown in Figure 2B. The product was identified by its ability to chelate ^{111}In(III) and its 360 MHz NMR spectrum (Figure 4). The latter shows, in addition to the resonances associated with Co(III) bleomycin, an ab pattern associated with the p-substituted benzene ring of the bifunctional chelating agent. As with BLEDTA I, the methyl resonance associated with the terminal amine sulfur is at 3.1 ppm, which indicates formation of a sulfonium ion (3). Previous work (3) has shown that Co(III)-bleomycin B_2, which differs from Co(III)-bleomycin A_2DM only in the "terminal amine" group, is not alkylated at all under these conditions; this provides further evidence that only the terminal amine thioether group of Co(III)-bleomycin A_2DM reacts with (S)-1-(p-bromoacetamido benzyl) EDTA.

It is worth emphasizing that the final product, BLEDTA II, contains a non-labile Co(III) ion coordinated to the metal binding site of the bleomycin half of the molecule, and a free (non-coordinated) EDTA group coupled to the terminal amine of bleomycin. This is shown in Figure 3. Coordination to Co(III) enhances the accumulation of bleomycin in Ehrlich ascites tumors in

mice (17), and may have a similar effect on the concentration of bleomycin in other types of malignancies. Transfer of Co(III) from the metal binding site of bleomcyin to the EDTA moiety is unlikely. In EDTA challenge experiments with ^{57}Co-bleomycin, Kono et al. (17) and Eckelman et al. (18) showed that Co(III) does not exchange; the results of our in vivo studies demonstrate that ^{111}In(III) must be binding only to the EDTA moiety of BLEDTA I (3).

Because the chelating moiety of BLEDTA II was prepared stereospecifically from L-phenylalanine, BLEDTA II differs from BLEDTA I stereochemically. Both BLEDTA I and BLEDTA II are mixtures of stereoisomers at the sulfonium ion sulfur, generated by the alkylation reaction. However, the chelating group of BLEDTA I is also a mixture of two stereoisomers, racemic with respect to the asymmetric carbon. Thus a BLEDTA I preparation contains 4 stereoisomers, while a BLEDTA II preparation contains only one pair of diastereomers. In accord with this, the NMR spectrum of BLEDTA II (Fig. 4) is simpler than that of BLEDTA I (3). Biological systems can be exquisitely sensitive to molecular configuration, but ^{111}In(III)-complexes of BLEDTAs I and II have identical in vivo distributions in mice (next section). Therefore concentration in neoplasms is not sensitive to the chiral center in the EDTA moiety.

In Vivo Studies in Tumor-bearing Mice

BALB/c mice with "KHJJ" tumor implanted in the flank were injected with ^{111}In(III)-BLEDTA II. Each of these mice also had a turpentine-induced abscess in the opposite flank. Figure 5 shows the organ distribution and tumor uptake of radioactivity in the mice; results of a similar experiment with ^{111}In(III) BLEDTA I are shown for comparison, since this compound has been found to have clinically useful tumor-localizing properties (3, 5).

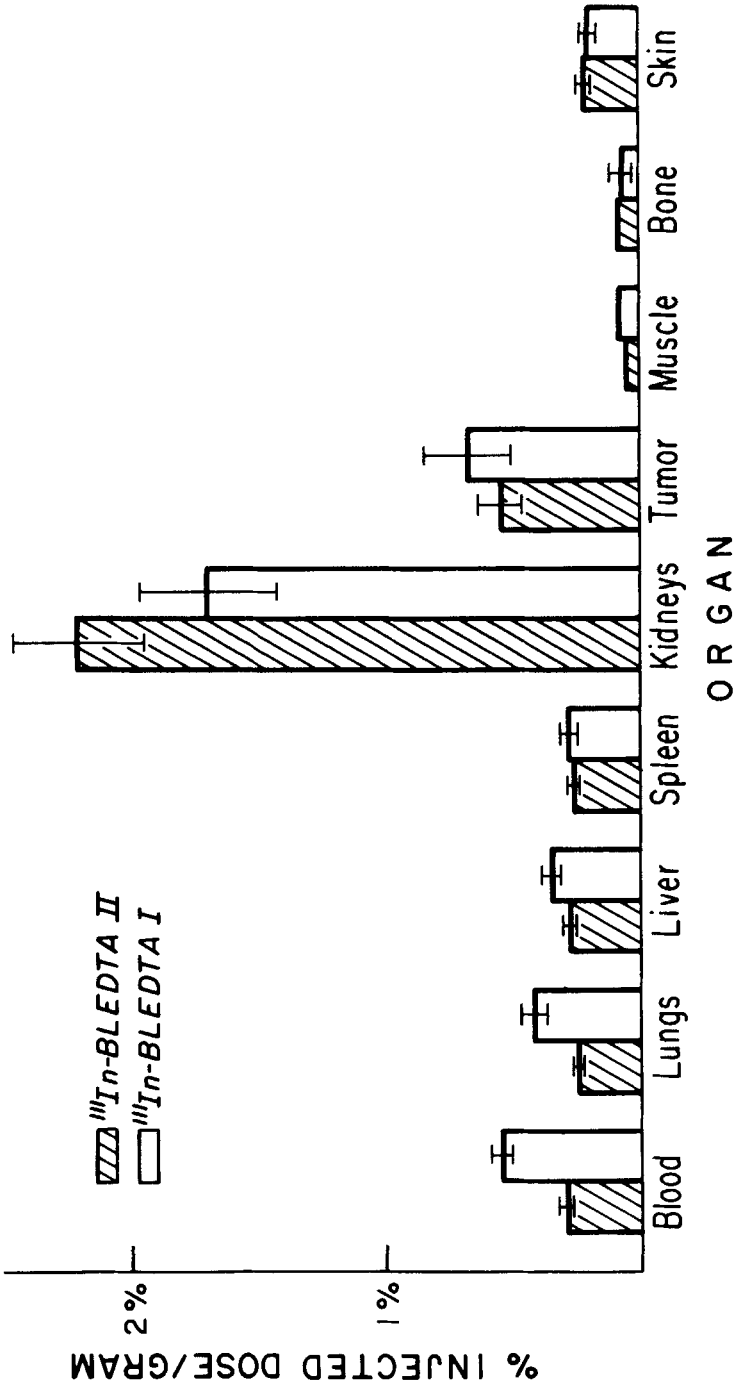

Figure 5. Organ distribution and uptake of ^{111}In(III)-BLEDTAs I and II in BALB/c mice with KHJJ Tumor. Unshaded, ^{111}In(III)-BLEDTA I (Mean ± SD for 3 mice); shaded ^{111}In(III)-BLEDTA II (Mean ± SD for 5 mice). The mice injected with ^{111}In(III)-BLEDTA II also had a turpentine-induced tumor in the opposite flank.

According to Figure 5, the in vivo distributions of radioactivity 24 h after injection of ^{111}In-BLEDTA I and II are nearly identical. Because the two compounds differ only by an extra methylene group in the terminal amine of BLEDTA II (Figure 1), this result is not surprising, and demonstrates that the more easily synthesized BLEDTA II is a useful diagnostic radiopharmaceutical.

Clinical studies with ^{111}In(III)-BLEDTA II have just begun. Because the ^{111}In(III) chelates of the two BLEDTAs have identical in vivo distributions in mice, it is likely that the clinical results obtained with ^{111}In(III)-BLEDTA II will be similar to those reported for ^{111}In(III)-BLEDTA I (5). Initial studies with human patients are in agreement with this expectation (D. A. Goodwin et al., unpublished results).

Recent evidence suggests that the BLEDTA terminal amine sulfonium group may be acting as a weak alkylating agent in vivo, transferring the radioactive chelate moiety to a nucleophile. This reactivity may explain the small (2% of the injected dose), but persistent background radioactivity found associated specifically with polymorphonuclear leukocytes after injection of ^{111}In(III)-BLEDTA, but not after injection of ^{57}Co(III)-bleomycin.

If the sulfonium group of BLEDTAs I and II is acting as an alkylating agent in vivo, its elimination should lower the levels of background radioactivity. We are trying two approaches to making a BLEDTA without a sulfonium group: demethylation of the sulfonium group of BLEDTA II, and preparation of a new BLEDTA via Co(III)-bleomycinic acid.

CONCLUSIONS

Versatile probes of biological systems can be prepared by coupling biologically interesting molecules to chelating agents. The results of clinical studies done with the ^{111}In(III) chelates of BLEDTA I and BLEDTA II

demonstrate the potential of this approach for practical applications. The technique is not limited to the preparation of ^{111}In radiopharmaceuticals; it can be used to tag molecules with a variety of other metal ions having useful physical properties.

REFERENCES

1. Umezawa H. - Pure Appl. Chem. 28:665 (1970).
2. Fujii A., Takita T., Maeda K. and Umezawa H. - J. Antibiot. 26:398 (1973).
3. DeRiemer L. H., Meares C. F., Goodwin D. A. and Diamanti C. I. - J. Med. Chem. 22:1019 (1979).
4. Meares C. F., DeRiemer L. H. and Goodwin D. A. - Bleomycin:Chemical, Biochemical and Biological Aspects (S. M. Hecht, Ed.) pp. 309-323, Springer Verlag, New York, 1979.
5. Goodwin D. A., Meares C. F., DeRiemer L. H., Diamanti C. I., Goode R. L., Baumert J. E. Jr., Sartoris D. J., Lantieri R. L. and Fawcett H. D. - submitted for publication.
6. Yeh S. M., Sherman D. G. and Meares C. F. - Anal. Biochem. 100:152 (1979).
7. Yeh S. M., Meares C. F. and Goodwin D. A. - J. Radioanal. Chem. 53:327 (1979).
8. Leung C. S.-H. and Meares C. F. - Int. J. Appl. Radiat. Isot. 29: 687 (1978).
9. Thiers R. - Methods Biochem. Anal. 5:273 (1957).
10. Leung C. S.-H. - Ph.D. Thesis, University of California, Davis, 1977, p. 113.

11. Beilsteins Handbuch der Organischen Chemie, B XIV, vierte auflage, Julius Springer, Berlin, 1931, p. 506.

12. Greenstein J. P. and Winitz M. - Chemistry of the Amino Acids, Wiley, New York, 1961 pp. 1188-1189.

13. Sundberg M. W., Meares C. F., Goodwin D. A. and Diamanti C. I. - J. Med. Chem. 17:1304 (1974).

14. Taube H. - Chem. Rev. 50:69 (1952).

15. Kakinuma J., Kagiyama R. and Orii H. - Eur. J. Nucl. Med. 5:159 (1980).

16. Vos C. M., Westera G. and van Zanten B. - J. Inorg. Biochem. 12:45 (1980).

17. Kono A., Matsushima Y., Kojima M. and Maeda T. - Chem. Pharm. Bull. 25:1725 (1977).

18. Eckelman W. C., Rzeszotaski W. J., Siegel B. A., Kubota H., Chelliah M., Stevenson J. and Reba R. C. - J. Nucl. Med. 16:1033 (1975).

19. Chen D. M., Hawkins B. L. and Glickson J. D. - Biochemistry 16:2731 (1977).

20. Friedman O. M. and Boger E. - Anal. Chem. 33:906 (1961).

21. Sugiura Y., Ishizu K. and Miyoshi K. - J. Antibiot. 32:453 (1979).

22. Dabrowiak J. C., Greenaway F. T., Longo W. E., Van Husen M. and Crooke S. T. - Biochim. Biophys. Acta 517:517 (1978).

23. Oppenheimer N. J., Rodriguez L. O. and Hecht S. M. - Biochemistry 18: 3439 (1979).

24. Oppenheimer N. J., Rodriguez L. O. and Hecht S. M. - Proc. Nat'l. Acad. Sci. USA 76:5616 (1979).