A Novel Method of Conjugation of Daunomycin with Antibody with a Poly-L-glutamic Acid Derivative as Intermediate Drug Carrier. An Anti-α-fetoprotein Antibody–Daunomycin Conjugate

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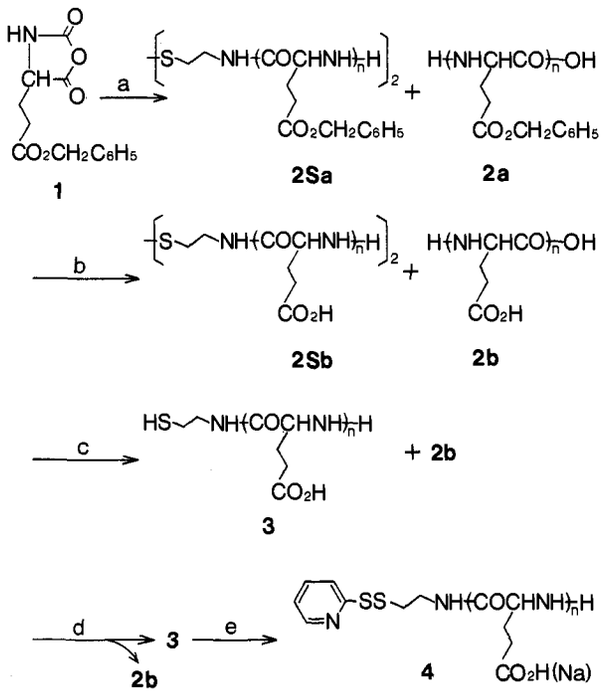
In studies on antitumor antibody–cytotoxic drug conjugates as potential antitumor agents with improved tumor specificity, daunomycin (DM) was first linked to a poly-L-glutamic acid (PLGA) derivative having a single masked thiol group. At the thiol group, DM-linked PLGA was bound to horse anti-rat α-fetoprotein (AFP) antibody. The anti-AFP antibody–PLGA–DM conjugate (anti-AFP conjugate, DM/PLGA/Ig molar binding ratio, 7.5/1.2/1.0) retained most of the antigen-binding activity of the parent antibody and was more potent than either unconjugated DM, a conjugate similarity prepared with normal horse immunoglobulin (normal conjugate), or an unconjugated mixture of anti-AFP antibody and DM in an in vitro cytotoxicity assay against the AFP-producing rat ascites hepatoma cell line AH66. Anti-AFP conjugate tended to be less cytotoxic than DM against the AFP-nonproducing rat ascites hepatoma AH272 cells, and in this case there was no difference between the cytotoxicities of anti-AFP conjugate and of normal conjugate.

A major problem of cancer chemotherapy is the lack of sufficient tumor selectivity of the cytotoxic antitumor drugs currently available. The nonspecific toxic action to normal cells limits the doses of the drugs to be administered to patients, resulting in unsatisfactory efficacy. One possible approach to alleviation of this problem is the conjugation of the drugs with antibodies to tumor-associated antigens expressed on tumor cells.[1-6] This theoretically should help to localize the drugs at the tumor sites.

However, the direct covalent binding of drugs to antibody with or without a spacer arm is often unsuccessful in making efficacious conjugates that have a defined chemical structure and are stable in aqueous solutions not readily precipitating. A daunomycin conjugate prepared with a water-soluble carbodiimide was inactive,[7] possibly because the amide bond between the amino group of daunomycin and the carboxyl group of antibody was insusceptible to enzymatic cleavage. Although another daunomycin conjugate with glutaraldehyde as the cross-linking agent was active, its preparation was accompanied by the formation of a considerable amount of aggregate due to the homo-coupling of antibody molecules.[7]

The primary objective of the present study was to develop a method of conjugation of amino group containing drugs, like daunomycin, with antibody to provide conjugates that exhibit antibody-directed antitumor activity and yet whose preparations are free from aggregates. An additional objective was to develop a method of conjugation by which a considerably large number of drugs can, in principle, be covalently conjugated with one molecule of antibody without significant detrimental effect on the antigen-binding activity of the antibody. We have developed such a method, which involves the use of a single thiol group containing poly-L-glutamic acid (PLGA) derivative as the intermediate drug carrier, and using this method we have prepared a conjugate of daunomycin (DM) with antibody to rat α-fetoprotein (AFP), which is produced by rat ascites hepatoma cells AH66 and is detectable on the cell surface as tumor-associated antigen.[8-10] The conjugate anti-AFP antibody (a-AFP)–PLGA–DM exhibited a greater cytotoxic activity to AH66 than did either unconjugated DM, a conjugate prepared similarly with normal horse immunoglobulin (nIg), or an unconjugated mixture of a-AFP and DM.

Scheme I



a, cystamine; b, CF_3CO_2H/CH_3SO_3H/anisole; c, DTT; d, Thiopropyl Sepharose 6B resin, 2-ME; e, 2-pyridyldisulfide.

Chemistry. In order to make a biologically active conjugate of DM with antibody, we first linked DM to a

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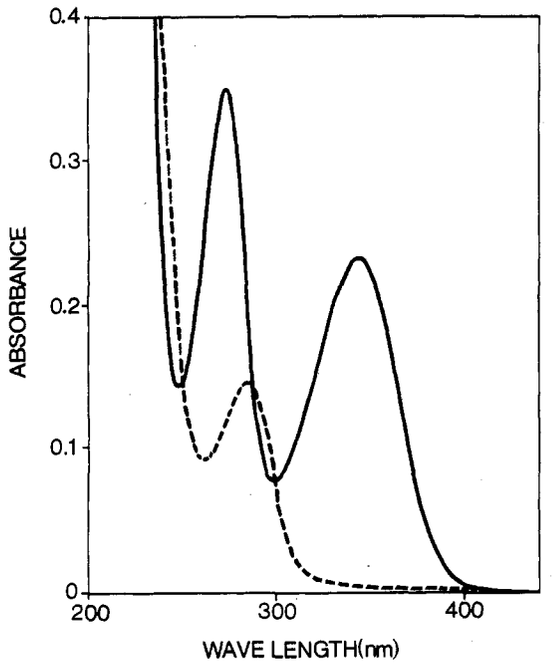

Figure 1. UV spectra of **4** before (---) and after (–) the treatment with DTT.

PLGA derivative having a single masked thiol group. This functional group was later selectively used for binding DM-linked PLGA to antibody. Since a typical method of making PLGA was the polymerization of γ-benzyl-*N*-carboxy-L-glutamate anhydride (1) with a base as initiator followed by debenzylation, we envisioned the preparation of a PLGA having a functional group equivalent to the thiol group by the polymerization of 1 with a sulfur atom containing amine (Scheme I).

Polymerization of **1** with cystamine gave the sulfur-containing and noncontaining poly-L-glutamic acid benzyl esters **2Sa** and **2a**.[11] Compound **2Sa** is the dimeric form of an ester of the final intermediate drug carrier (**3**) having a single thiol group. The mixture of **2Sa** and **2a** was treated with trifluoroacetic acid and methanesulfonic acid to give a mixture of the free carboxylic acids **2Sb** and **2b**. The acid **2b** thus derived from the byproduct **2a** could be removed with Thiopropyl-Sepharose 6B resin. The resin contains active disulfide 2-pyridyldithio groups and reacts selectively with thiol compounds that can be recovered from the resin through the disulfide exchange reaction with 2-mercaptoethanol (2-Me) or dithiothreitol (DTT). Treatment of the mixture of **2Sb** and **2b** with DTT followed by removal of **2b** by the use of Thiopropyl-Sepharose 6B resin afforded the drug carrier **3**, which was stored in the form of the disulfide **4**. The average molecular weight of **4** was determined to be 12000 by treatment of an aliquot of **4** with DTT followed by measurement of the absorbance at UV absorption maximum at 343 nm due to liberated pyridine-2-thione (Figure 1).

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(10) Mizejewski, G. L.; Allen, R. P. *Clin. Immunol. Immunopathol.* **1978**, *11*, 307–317.
(11) The presence of the sulfur-noncontaining polymer **2a** in the product was known as follows: the content of the disulfide group as determined with respect to an aliquot of the debenzylated product by reduction of the disulfide group to the thiol group followed by the quantitation of the thiol group by the method of Ellman (Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *32*, 70–77.] was 62% of the theoretical as calculated by deviding the weight of the lyophilized sample by the molecular weight obtained by viscometry.

Scheme II

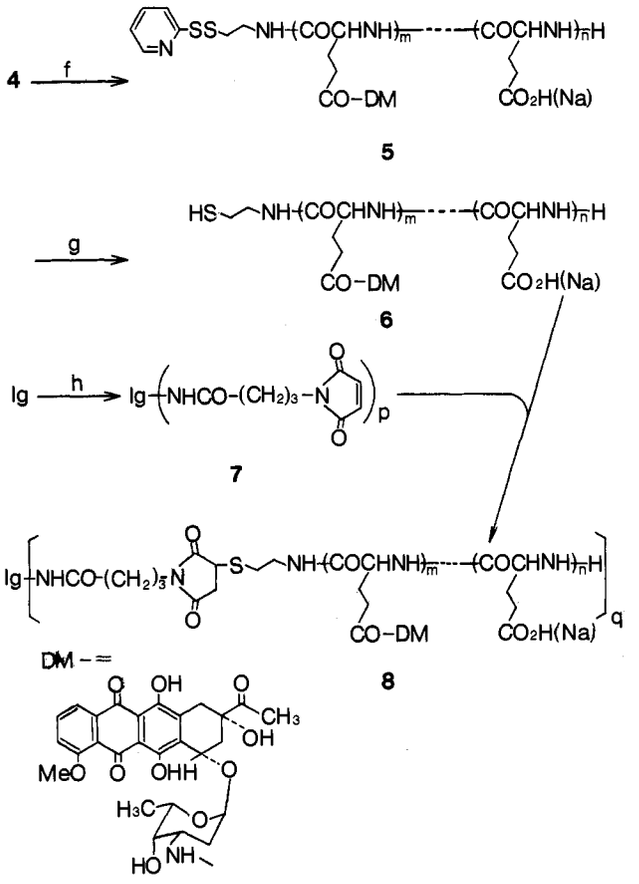

Table I. Chemical Data of Ig-PLGA-DM Conjugates

	conjugate	
	anti-AFP conjugate	normal conjugate
PLGA		
average M_r[a]	12 000	
degree of polymerization	80	
PLGA-DM		
DM-to-PLGA molar binding ratio[b]	6.5	
drug-substitution rate, %	8.2	
Ig-PLGA-DM		
PLGA-to-Ig binding ratio[c]	1.2	1.1
DM-to-Ig binding ratio[d]	7.5	7.1
purity of the preparation subjected to in vitro cytotoxicity studies,[e] %	90	93

[a] Determined by the end-group (2-pyridyldithio) analysis of **4**. [b] The DM content was determined spectrophotometrically and the PLGA content was determined by the end-group analysis. [c] Calculated by dividing the DM-to-Ig binding ratio by the DM-to-PLGA binding ratio. [d] Obtained from mole of Ig in the conjugate preparation [determined by the Bio-Rad protein assay (ref 24)] and mole of Ig-linked DM as determined by multiplying the total DM content (Ig-linked DM plus DM as PLGA-DM) of the conjugate preparation (determined by UV absorbance measurement) by $p/100$ [p, purity (percent) of the conjugate: percentage of Ig-linked DM in total amount of DM]. [e] Purity was determined as follows: the conjugate preparations were subjected to disc PAGE, and Ig-PLGA-DM and PLGA-DM were isolated from the corresponding bands by further electrophoresis. The contents of DM extracted in the above two forms were determined spectrophotometrically (λ 480 nm).

DM was linked to the PLGA derivative **4** with use of the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI) to give

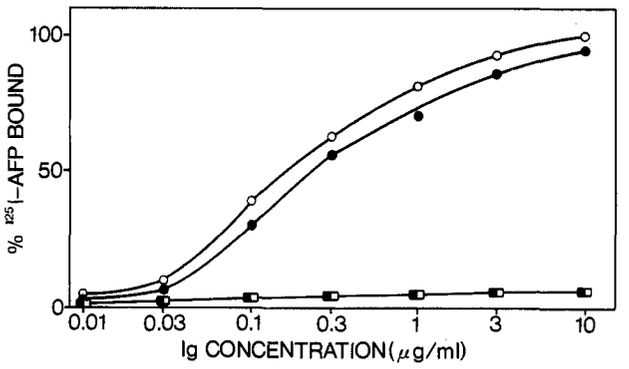

Figure 2. Antigen-binding activity of a-AFP–PLGA–DM. The antigen-binding activity was determined by the following procedure (ref 29). ^{125}I-labeled rat AFP with a specific activity of 3.6×10^7 cpm/nmol [ref 30 (appendix, procedure B)] in PBS containing 10% horse serum (70 μg/mL, 100 μL) was mixed with a-AFP (○), a-AFP–PLGA–DM (●), nIg (□), or nIg–PLGA–DM (■) in PBS containing 10% horse serum (0.01–10 μg/mL, 100 μL), and the mixture was incubated with gentle shaking for 24 h at room temperature. Then, 21% polyethylene glycol in PBS (1 mL) was added, and after having been allowed to stand for 15 min, the mixture was centrifuged at 2000 rpm for 10 min. The supernatant was removed and the radioactivity in the precipitate was counted in a gamma counter. The radioactivity in the precipitate generated from 10 μg/mL of a-AFP and ^{125}I-labeled rat AFP is expressed as 100%.

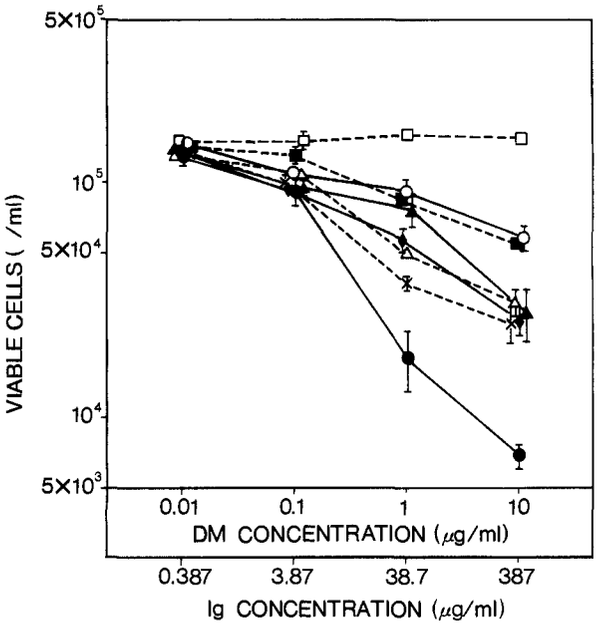

Figure 3. Cytotoxicity of a-AFP–PLGA–DM against AH66 cells. AH66 cells (5×10^4 cells/mL, 0.2 mL) were cultured at 37 °C for 48 h with various concentrations of either nIg (□), a-AFP (○), DM (x), PLGA–DM (△), mixture of a-AFP and DM (▲), mixture of a-AFP, and PLGA–DM (♦), nIg–PLGA–DM (■), or a-AFP–PLGA–DM (●), and the numbers of the viable cells were determined. Points, mean of triplicate determinations; bars, SE (indicated unless smaller than the points as plotted). For details, see the Experimental Section.

the PLGA–DM conjugate **5** (Scheme II). The average number of DM molecules linked to one molecule of PLGA (DM-to-PLGA binding ratio) and the ratio (percent) of the number of drug-substituted carboxyl groups to the total number of drug-substituted and unsubstituted carboxyl groups (drug-substitution rate) in **5** are shown in Table I.

The final conjugation was achieved by the addition reaction of the thiol group of the DM-linked PLGA derivative **6** (the thiol group was generated from **5**) with the maleimide-modified a-AFP and nIg. For the introduction of the maleimide groups, the immunoglobulins were treated with a 10-fold molar excess of *N*-succinimidyl 4-(*N*-maleimido)butyrate (SMBU) resulting in the introduction of 6.8 (a-AFP) and 6.5 (nIg) maleimide groups per molecule of immunoglobulin as determined by an assay with *N*-(2,4-dinitrophenyl)cysteine (DNP-Cys). The modified immunoglobulins **7** were then treated with a 4-fold molar excess of **6**, and the conjugates **8** were purified by large-scale disc polyacrylamide gel electrophoresis (PAGE). The purity of the conjugate preparations thus obtained [percentage of immunoglobulin-linked DM in total amount of DM (immunoglobulin-linked DM plus DM as PLGA–DM)], the average number of PLGA linked to one molecule of Ig (PLGA-to-Ig molar binding ratio), and the average number of DM molecules linked to one molecule of Ig through PLGA (DM-to-Ig molar binding ratio) of the conjugates are shown in Table I.

Biology. The antigen-binding activity of a-AFP and its DM conjugate was determined with use of ^{125}I-labeled AFP. The results depicted in Figure 2 demonstrate that the antibody retained most of the original binding activity after conjugation of DM via PLGA.

The cytotoxic activity of a-AFP–PLGA–DM conjugate against the AFP-producing rat ascites hepatoma AH66 cells[12] was assessed by determining the inhibition of the in vitro growth of AH66 cells (Figure 3). This was compared to the inhibitions with nIg, a-AFP, unconjugated DM, PLGA–DM, a mixture of a-AFP and DM, a mixture of a-AFP and PLGA–DM, and nIg–PLGA–DM.

Anti-AFP conjugate was more cytotoxic against AH66 cells at 1 and 10 μg (equivalent DM)/mL than was either a-AFP ($P < 0.1$), DM ($P < 0.05$), a mixture of a-AFP and DM ($P < 0.05$), a mixture of a-AFP and PLGA–DM ($P < 0.01$), or normal conjugate ($P < 0.001$). These results suggest that, for a potent cytotoxicity, a-AFP (antibody activity) and DM (drug activity) are both necessary and that they must be covalently linked with each other.

Next, a similar in vitro cytotoxicity assay was performed by using the AFP-nonproducing rat ascites hapatoma AH272 cells. In this case, contrary to the case with AH66, anti-AFP conjugate tended to be less cytotoxic than DM and there was no difference in cytotoxicity between anti-AFP conjugate and normal conjugate. A considerably large cytotoxicity of anti-AFP conjugate against the AFP-nonproducing AH272 is probably due to the fact that AH272 is considerably more sensitive to DM than is AH66 as shown in Figure 4. Anti-AFP and normal conjugates were probably taken up by the cells by nonspecific endocytic internalization as was the case[13] with macromolecular drug derivatives. Not only nIg but also a-AFP showed no cytotoxicity against AH272 cells.

Discussion

Our choice of a PLGA derivative as the intermediate drug carrier in making a conjugate of DM with a-AFP was dictated by the fact that PLGA is water soluble, is biodegradable, and has many carboxyl groups that can be used for the covalent linking of DM at its sugar amino group.

PLGA itself was previously used as the intermediate drug carrier in conjugating *N*,*N*-bis(2-chloroethyl)-*p*-

(12) The AH66 cells (initial cell number, 5×10^4 cells/mL) produce ca. 570 μg of AFP/mL after 48-h in vitro culture under conditions described in the Experimental Section.

(13) De Duve, C.; De Barsy, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F. *Biochem. Pharmacol.* 1974, *23*, 2495–2531.

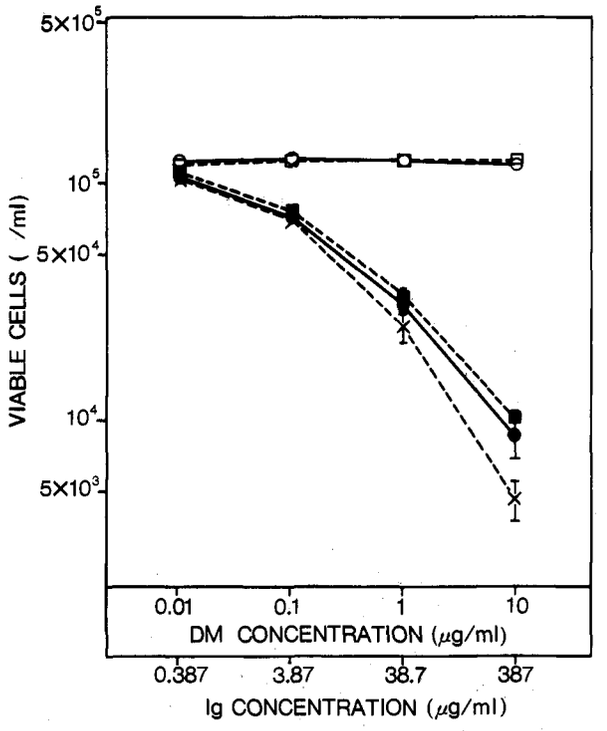

Figure 4. Cytotoxicity of a-AFP–PLGA–DM against AH272 cells. AH272 cells (5×10^4 cells/mL, 0.2 mL) were cultured at 37 °C for 48 h with various concentrations of either nIg (□), a-AFP (○), DM (x), nIg–PLGA–DM (■), or a-AFP–PLGA–DM (●), and the numbers of the viable cells were determined. Points, mean of triplicate determinations; bars, SE (indicated unless smaller than the points as plotted). For details, see the Experimental Section.

phenylenediamine, an alkylating agent, with antibody to the murine lymphoma EL4.[14] However, in this previous work the carboxyl groups of PLGA were used not only for binding drug to PLGA but also for binding drug-linked PLGA to antibody. These two binding reactions were both effected with the carbodiimide EDCI. The drug-linked PLGA still contains many free carboxyl groups which allow more than one molecule of antibody to bind to the PLGA chain. Use of EDCI for binding the drug-linked PLGA to antibody also effects the homo-coupling of the antibody molecules leading to the formation of a heterogeneous high-molecular-weight aggregate. Thus, when the amount of EDCI was large enough to achieve coupling of the drug-linked PLGA to the antibody, it was difficult to prevent homo-coupling of the antibody itself.[15] The present method of conjugation using a PLGA derivative as the intermediate drug carrier is unique in the following respects: (1) the drug carrier has a single thiol group to bind to antibody and a large number of carboxyl groups to bind drug molecules, (2) for the former binding a selective reaction was used in which the thiol group of the carrier was allowed to react with maleimide-modified antibody. The resulting conjugate has a well-defined chemical structure.

Anti-AFP conjugate 8 retained most of the antigen-binding activity of the parent antibody and was cytotoxic against the AFP-producing rat ascites hepatoma AH66 cells. It was more cytotoxic than unconjugated DM. The observation that the anti-AFP specific conjugate is more active than either normal conjugate or an unconjugated mixture of a-AFP and DM suggests that the binding of the antibody portion of the conjugate to the AFP molecules on AH66 cells is involved for the cytotoxic activity of anti-AFP conjugate.

When the AFP-nonproducing rat ascites hepatoma AH272 cells were used as target instead of AFP-producing AH66, anti-AFP conjugate tended to be less cytotoxic than DM and the difference in cytotoxicity observed against AH66 between anti-AFP conjugate and normal conjugate was no longer observed. DM itself was more cytotoxic against AH272 cells than against AH66 cells and this would explain the considerably large cytotoxic action even against AH272 cells of anti-AFP conjugate, which was probably internalized by nonspecific endocytosis of AH272 cells.

Although it was reported that an anti-mouse B leukemia antibody–DM conjugate prepared by direct conjugation with a carbodiimide as condensation agent was almost inactive against the B leukemia cells, the present a-AFP conjugate was active against AH66 cells. The precise mode of action of a-AFP conjugate after possible targeting to the tumor cells is unclear, but it may be speculated that the activity is due to the release of small-molecular-weight active DM derivatives and/or DM itself through the degradation of the PLGA chain in lysosomes.[13,16]

It is known that the number of drug molecules that can be linked covalently to an antibody without significantly decreasing its antibody activity is very limited.[7] The method of conjugation developed in the present study using **3**, a novel derivative of PLGA, as the intermediate drug carrier has the potential to considerably increase the number of DM molecules per antibody. The polymer **3** should be a versatile intermediate drug carrier for conjugation to antibody.

Experimental Section

UV spectra were recorded on a Hitachi Model 200-20 spectrophotometer. NMR data were obtained on a Varian EM360A spectrometer. Viscometry was done with a Ubbelohde viscometer (Shibata Chemical Appliance, Tokyo).

***N,N'*-Bis(γ-benzylpoly-L-glutamyl)cystamine (2Sa).** To a stirred solution of γ-benzyl-*N*-carboxy-L-glutamate anhydride[17] (1; 7.75 g, 29.4 mmol) in dry dioxane (185 mL) was added cystamine (95 mg, 0.62 mmol) dissolved in dry dioxane (10 mL). After stirring at room temperature for 24 h, the mixture was poured into stirred isopropyl ether (4 L). The precipitated material was collected by filtration and dried to give a mixture of **2Sa** and γ-benzylpoly-L-glutamate (**2a**) [average M_r determined[18] from $[\eta]$ ($CHCl_2CO_2H$, 25 °C), 47 300]; 6.19 g, 96%.

***N,N'*-Bis(poly-L-glutamyl)cystamine (2Sb).** To a solution of the above mixture of **2Sa** and **2a** (3.11 g) in a mixture of trifluoroacetic acid (25 mL) and anisole (4.5 mL) was added methanesulfonic acid (25 mL), and the mixture was stirred for 20 min on an ice bath and for 30 min at room temperature. The mixture was poured into isopropyl ether (450 mL). The precipitated material [**2Sb** and poly-L-glutamic acid (**2b**)] was collected by filtration and suspended in water (50 mL). The suspension was stirred with saturated $NaHCO_3$ solution (60 mL) to give a colorless solution. The solution was dialyzed against water and lyophilized to give Na salts of **2Sb** and **2b** as colorless material [average M_r as determined[19] from $[\eta]$ (0.01 M sodium phosphate buffer, pH 7.0, ionic strength I 0.11, containing 0.1 M NaCl, 25.5 °C), 29 200]; 1.91 g, 89%. Removal of the benzyl group was confirmed by NMR [D_2O, δ from 3-(trimethylsilyl)propanesulfonic

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(19) Hawkins, R. B.; Holtzer, A. *Macromolecules* **1972**, *5*, 294–301.

acid (Na salt)]; no signal (the phenyl group) was observed between δ 6.0 and 10.0.

2-(Poly-L-glutamylamino)ethyl 2-Pyridyl Disulfide (4). To a solution of the Na salts of **2Sb** and **2b** (126 mg, 4.32 μmol) in 0.1 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA (10 mL) was added DTT (15.4 mg, 0.1 mmol), and the mixture was stirred for 1 h at 50 °C and dialyzed against 0.1 M sodium phosphate buffer, pH 6.0, containing 1 mM EDTA. The solution thus obtained was added to a suspension of Thiopropyl-Sepharose 6B resin [(Pharmacia Fine Chemicals, Uppsala, Sweden; 26 mL (wet volume)] in 0.1 M sodium phosphate buffer, pH 6.0, containing 1 mM EDTA (50 mL), and the mixture was stirred for 12 h. The resin was collected by filtration, suspended in 0.1 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA (40 mL), and stirred gently for 12 h with 2-ME (2.03 g, 26 mmol). The mixture was filtered, and the resin was washed with the above Tris-HCl buffer. The filtrate and washings were combined, acidified to pH 1.9 with 1 N HCl, and allowed to stand at 4 °C for 2 days. The generated precipitate [2-(poly-L-glutamylamino)ethanethiol (**3**)] was collected by centrifugation.

A solution of 2-pyridyl disulfide (PDS) was made by adding PDS (57 mg, 259 μmol) dissolved in ethanol (5 mL) to 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA (20 mL). Thiol **3** was dissolved in 0.4 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA (5 mL), and to the resulting solution was added the ice-cold PDS solution. The mixture was allowed to stand at room temperature for 30 min and, after removal of a small amount of insoluble material by filtration, was dialyzed against water and lyophilized to give **4** [average M_r as determined from the end-group (2-pyridyldithio group) analysis, 12 000]; 39 mg, 31%.

Binding of DM to 4. To a solution of **4** (30 mg, 2.5 μmol, 0.2 mmol) in 2% aqueous NaCl solution (15 mL) was added 3.55 mM aqueous solution of DM·HCl (8.45 mL, 30 μmol), and the pH of the solution was made 5.5 with 1 N HCl. To the resulting solution was added EDCI (38.3 mg, 0.20 mmol), and the mixture was stirred at room temperature for 18 h. After 0.4 M sodium phosphate buffer, pH 7.0 (5 mL), was added to the solution, a small amount of precipitate was removed by centrifugation. An aliquot (0.5 mL) of the solution was put aside, and the rest was dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and against water. The resulting solution was evaporated at 40 °C under reduced pressure to ca. 6 mL and dialyzed against 10 mM sodium phosphate buffer, pH 7.0, to give a solution (8 mL) of the PLGA–DM conjugate **5**. The DM content of the solution of **5** thus obtained was determined by UV absorbance measurement (15.0 μmol) and the PLGA content by the end-group analysis (2.30 μmol). On the basis of these data, yields of **5** in terms of DM and PLGA were 50% and 93%, respectively. The DM-to-PLGA molar binding ratio and the drug-substitution rate were shown in Table I.

Binding of PLGA–DM to Immunoglobulins. To a solution of **5** in 10 mM sodium phosphate buffer, pH 7.0 (0.27 mM of PLGA equivalence, 2.20 mL), was added 0.30 M aqueous DTT solution (0.1 mL). The mixture was allowed to stand at 40 °C for 30 min and then dialyzed against 0.1 M sodium phosphate buffer, pH 6.0, to give DM-linked PLGA having a single thiol group (**6**).

A 32.3 mM solution of SMBU in *N,N*-dimethylformamide (DMF) (0.05 mL) was added to a solution of a-AFP in 10 mM sodium phosphate buffer, pH 7.0, containing 0.14 M NaCl (20.0 mg/mL, 1.21 mL), and the mixture was allowed to stand at 25 °C for 1 h and subjected to gel filtration on a Sephadex G-25 column (0.8 × 44 cm) with 0.1 M sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl.

To the resulting solution of the maleimide group containing a-AFP [**7** (Ig = a-AFP); 5.04 mg/mL, 3.00 mL] was added the solution of **6** prepared as described above (0.204 mM of PLGA equivalence, 1.98 mL), and the mixture was allowed to stand at 25 °C for 26 h to give a crude solution of the a-AFP–PLGA–DM conjugate **8** (Ig = a-AFP).

Purification of the conjugate was performed by preparative disc PAGE. The crude product was applied to a gel (5% polyacrylamide, 2.0 × 6.0 cm), and electrophoresis was conducted in 5 mM Tris–38 mM glycine buffer, pH 8.6, at 30 mA for 3 h to separate the conjugate from unreacted **6**. The portion of the gel containing the conjugate that migrated slowly[20] was cut out, and the conjugate was isolated from the gel by further electrophoresis. This purification procedure was repeated once more to give the conjugate a-AFP–PLGA–DM.

A corresponding conjugate of DM with nIg (Cappel Laboratories, Cochranville, PA) was prepared by the same procedure.

The purity of the anti-AFP and normal conjugates prepared are shown in Taable I. The PLGA-to-Ig and DM-to-Ig molar binding ratios of the conjugates are also shown in Table I together with the methods of determination.

Quantitation of DM. The DM content was determined spectrophotometrically based on absorbance at λ 480 nm (ε 12 000).[21]

Quantitation of the 2-Pyridyldithio End Group of PLGA. The content of the 2-pyridylthio end group was determined by treatment of a sample with DTT followed by measurement of UV absorbance at λ_{max} 343 nm (ε 8080)[22] due to liberated pyridine-2-thione. A sample was dissolved in or diluted to 2–3 mL of 0.1 M sodium phosphate buffer, pH 70, and treated with an excess (10 μL) of 10 mM DTT in 0.1 M sodium phosphate buffer, pH 7.0, and absorbance at 343 nm was measured.

Quantitation of the Maleimide Group Introduced to Ig. An aliquot of a sample (ca. 2.5 mg) was treated with ca. 50-fold molar excess of DNP-Cys in 0.1 M sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl at room temperature for 1 h, and unreacted DNP-Cys was removed by gel filtration on a Sephadex G-25 column (0.8 × 40 cm) in phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.9 mM $CaCl_2$; 0.5 mM $MgCl_2$, pH 7.2). UV absorption at λ_{max} 360 (ε 17 000)[23] and 280 nm ($E_{1cm}^{1\%}$ 14.0) was measured for determination of the content of the maleimide group and of the protein.

Quantitation of Ig in Conjugates. Quantitation of Ig protein was performed by Bio-Rad protein assay[24] (Bio-Rad Laboratories, Richmond, CA), which was based on the shift of absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 from 465 to 595 nm when binding to protein occurred. A standard curve was drawn with use of nIg, and correction was made for the effect of PLGA–DM not conjugated to the antibody.

Anti-AFP Antibody. Specific antiserum to rat AFP was produced in a horse by weekly subcutaneous injections of 1 mg of purified AFP emulsified in Freund's complete adjuvant.[25] Anti-AFP antibody[26] was purified by affinity chromatography of the antiserum on activated Sepharose 4B coupled to rat AFP.[27]

Tumor Cells. The rat ascites hepatoma cells AH66 (AFP-producing) and AH272 (AFP-nonproducing) used in this study were maintained by intraperitoneal passage in syngeneic male Donryu rats (Nihon Rat, Saitama).[8,28]

In Vitro Cytotoxicity Test. AH66 or AH272 cells were cultured in Eagles minimal essential medium (pH 7.4) (Nissui Seiyaku, Tokyo) (5 × 10^4 cells/mL, 0.2 mL) containing 10%

(20) Bands are visible by the red color. The band of the conjugate was known as such from the result of an analytical small-scale disc PAGE run separately.

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heat-inactivated calf serum (Flow Laboratories, Stanmore, Australia), kanamycin sulfate (6 μg/mL), and a serially diluted test sample in a 96-well microtest tissue culture plate (Falcon no. 3040) in a humidified atmosphere of 5% CO_2 in air at 37 °C for 48 h, and the viable cells were counted by the Trypan Blue dye exclusion method. Means of triplicate determinations were compared to respective controls by Student's t test.

Registry No. 1, 3190-71-4; **2a**, 25014-27-1; **2a** SRU, 25038-53-3; **25a**, 92694-88-7; **2b**, 25513-46-6; **2b** SRU, 24991-23-9; **2b**·Na, 26247-79-0; **25b**, 92694-90-1; **25b**·Na, 92694-91-2; **3**, 92694-86-5; DTT, 3483-12-3; PDS, 2127-03-9; cystamine, 51-85-4.

Synthesis of 4-Substituted 2*H*-Naphth[1,2-*b*]-1,4-oxazines, a New Class of Dopamine Agonists

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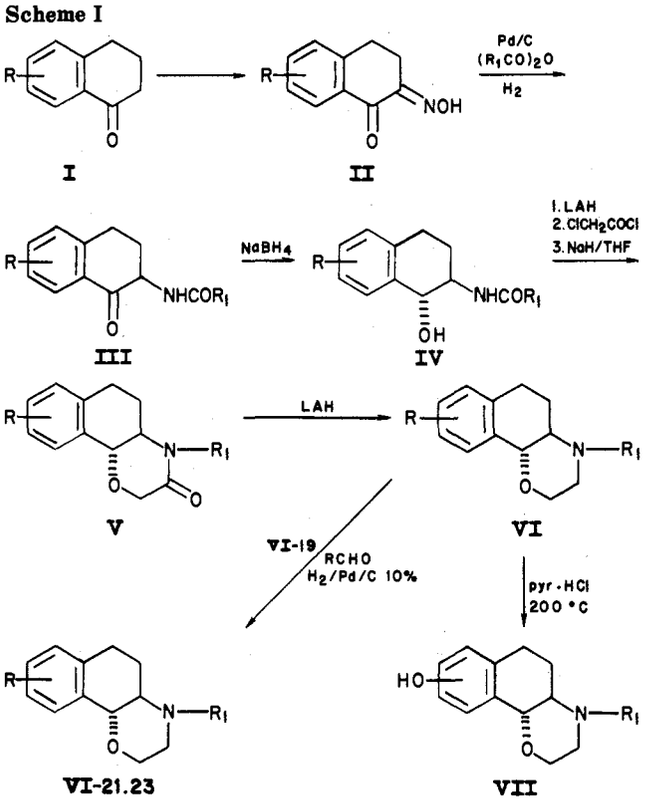
A series of tricyclic oxazines, namely, the 4-substituted 2*H*-naphth[1,2-*b*]-1,4-oxazines, have been synthesized and assayed for dopamine agonist activity. One of the members of this series, compound (+)**VII-15**, was found to be a remarkably potent agonist in vivo when tested in the standard 6-hydroxydopamine lesioned rat assay. The absolute configuration of the compound corresponds to that found in the active isomer of apomorphine. Its activity at the α_2 receptor (vs. [^{3}H]clonidine) is relatively low. It also failed to stimulate the synthesis of cAMP in the carp retina assay, thus giving the compound a highly selective profile in favor of the D_2 receptor.

A direct-acting dopamine agonist with selectivity for the D_2 receptor[1] would have significant therapeutic utility in the treatment of Parkinson's disease. The classical examples for such an agent are apomorphine and the ergolines. Through the work of Cannon[2] and McDermed[3] and others,[4] it is known that many molecules that can be viewed as partial structures of these complex alkaloids have potent dopaminergic activity. We have recently reported[5] the synthesis of a new class of *D*-heteroergolines, the 9-oxaergolines. Partial structures related to these oxaergolines, namely, the naphth[1,2-*b*]-1,4-oxazines, have been prepared and are reported here to be dopamine receptor agonists. The most potent member of this series (+)-**VII-15** was examined by X-ray analysis[6] and its absolute configuration was found to be 1a*R*,4a*R*. The computer-generated ORTEP drawing of this molecule is presented in Figure 1. This is consistent with the chirality of the active isomer of apomorphine. Additionally, we have found (+)**VII-15** to have selectivity for the dopamine receptor vs. the α_2 receptor and the compound failed to stimulate cAMP synthesis when tested in the carp retina assay.

Chemistry. The synthetic strategy used for construction of the oxazine ring system was described in the first paper of this series[7] and is shown in Scheme I. Various tetralones were successfully annulated by using this method. Ether cleavage of the methoxynaphth[1,2-*b*]-1,4-oxazines using pyridine hydrochloride (Scheme I, step 5)

Scheme I



or several other methods failed for the 8- and 10-methoxy derivatives, necessitating the use of the benzyl protecting group, which was removed by catalytic hydrogenation to the desired phenols (Scheme II). Medium-pressure chromatographic separation[8] of the enantiomeric *l*-*O*-methylmandelate esters[7] of **IV-11** (Scheme III) provided the optical isomers (+)**VII-15** and (−)**VII-16**. Reductive alkylation (Scheme I, step 6) of **VI-19** afforded the N-substituted derivatives **VI-21,23**. The cis isomer **VII-18** was derived from medium-pressure chromatographic separation[8] of the mixture of cis and trans isomeric alcohols formed in the sodium borohydride reduction of ketone

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