Conjugation of Poly(styrene-*co*-maleic acid) Derivatives to the Antitumor Protein Neocarzinostatin: Pronounced Improvements in Pharmacological Properties

Hiroshi Maeda,*† Minoru Ueda,‡ Tetsuo Morinaga,† and Tomonori Matsumoto§

Department of Microbiology, Kumamoto University Medical School, Kumamoto 860, Central Research Laboratories, Kuraray Co., Ltd., Sakazu, Kurashiki 710, and Pharmacology Center, Kayaku Antibiotics Research Laboratories Co., Ltd., Iruma-Gun, Saitama 354, Japan. *Received February 6, 1984*

An anticancer agent of intermediate molecular weight and having both a hydrophilic and hydrophobic nature was developed by utilizing the antitumor protein neocarzinostatin (NCS; M_r = 12000) as a prototype drug. The modification was achieved by reacting the two amino groups on NCS with an anhydride group of partially half-esterified (p-E-) or partially hydrolyzed (p-H-) poly(styrene-*co*-maleic anhydride) (SMA) in 0.8 M $NaHCO_3$. The SMA samples with narrow molecular weights distributions (M_w = ca. 2000) were prepared by copolymerizing styrene and maleic anhydride in cumene followed by fractionation by means of a column-elution method. The derivatives p-E- or p-H-SMA were then formed by using the appropriate monoalcohols or H_2O, respectively. These SMA derivatives contain about 2 mol of anhydride residues/mol of SMA. The reaction product, SMA-conjugated NCS (designated as SMANCS), was purified by dialysis followed by gel filtration with Sephadex G-75. The complete reaction yielded essentially a single product, biantennary SMANCS. The molecular weight of the pure SMAMCS was estimated by various methods, including polyacrylamide gel electrophoresis with $NaDodSO_4$, HPLC in the gel permeation mode, fluorescence polarization, and a decrease in both nitrogen and protein contents. These results agree with the apparent molecular weight of about 16000. Characters of SMANCS was considerably altered from that of parental NCS: solubility characteristics in both organic and aqueous solvents were changed, the biological half-life in blood was prolonged 10 times, and antitumor activity became more pronounced, but the toxicity was reduced to one-fourth of the parental NCS. Thus, the present study has provided a method of improving biologically active substances by polymer conjugation.

Most of the known drugs in clinical use have had molecular weights of less than 1500. Recently, the use of drugs of a higher molecular weight range (between 10000 and 50000), such as interferons, plasma components, anticancer drugs including neocarzinostatin (NCS), and others, is opening up a new field. All of the drugs are common because they are protein. However, because of this protein nature, a few of their pharmacological properties are disadvantageous, such as short half-life in vivo and antigenicity. A vascular permeability of these drugs may be also unique due to their molecular size. These limitations or characteristics are all different from those of the low-molecular-weight drugs. NCS is a proteinaceous anticancer agent used for treatment of limited types of cancer including cancers of the bladder, the stomach, and leukemia.[2a] Its primary action is strand scission of DNA in cells.[2b,3,5a] In a structural study, NCS was shown to have only two free amino groups in a single polypeptide chain of 12000 daltons[4] cross-linked by two disulfide bridges intramolecularly.[5,6] The limitations of NCS for general clinical use in cancer chemotherapy include its short half-life[2] and, although common to many other anticancer agents, its toxicity. The toxicity of anticancer agents will be decreased if the drugs are delivered mainly to the tumor tissues rather than being distributed throughout the whole body in vivo. A partial improvement in pharmacological properties was accomplished by succinylation of amino groups on NCS:[7,8] there was no alteration in its activity at the molecular level but an improvement in antitumor activity in vivo. The present study is an extension along this line.

Chemical modification of protein has been an indispensable tool for the study of structure–activity relationships. The present investigation was undertaken to explore the use of chemical modification to overcome some of the above-described shortcomings of proteinaceous drugs. We describe here the synthesis of the copolymer of styrene and maleic anhydride (SMA) for this purpose and quantitative modification of the amino group of NCS with this copolymer. The results on the conjugate [SMANCS] indicate that, although the molecular mechanism of action of NCS remains unchanged,[9] the pharmacological properties in vivo can be greatly improved by this approach. Namely, much improved distribution in the lymphatics[9] and par-

† Kumamoto University.
‡ Kuraray Co., Ltd.
§ Kayaku Antibiotics Research Laboratories Co., Ltd.

(1) Abbreviations: NCS, neocarzinostatin; SMA, poly(styrene-*co*-maleic anhydride); p-H-SMA, partially hydrolyzed SMA; p-E-SMA, partially half-esterified SMA; p-Bu-SMA, partially half-butyl-esterified SMA; p-EtCe-SMA, SMA partially half-esterified with ethylcellosolve; SMANCS, NCS conjugated with p-H- or p-E-SMA; H-SMANCS, Bu-SMANCS, and EtCe-SMANCS, SMANCS prepared from NCS and p-H-SMA, p-Bu-SMA, or p-EtCe-SMA; HPLC, high-pressure liquid chromatography; GF, gel filtration; FP, fluorescence polarization; TNBS, trinitrobenzenesulfonic acid; VPO, vapor pressure osmometer; PAGE, polyacrylamide gel electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $\bar{M}_n$, number-average molecular weight; $(\bar{M}_n)_s$, $\bar{M}_n$ for SMA and its derivatives; $\bar{M}_w$, weight-average molecular weight; $(\bar{M}_w)_s$, $\bar{M}_w$ for SMA and its derivatives; $(\bar{M}_n)_{th}$, theoretical $\bar{M}_n$ value; $(\bar{M}_w)_{th}$, theoretical $\bar{M}_w$ value; LD_{50}, 50% lethal dose.

(2) (a) Maeda, H. *Anticancer Res.* **1981**, *1*, 175. (b) Ono, Y.; Watanabe, Y.; Ishida, N. *Biochim. Biophys. Acta* **1966**, *119*, 46.
(3) Ishida, R.; Takahashi, T. *Cancer Res.* **1978**, *38*, 2617.
(4) A recent investigation on the amino acid sequence of NCS fragment by FAB-MS showed four more amino acid residues (Asp, Thr, Ser, Ala) with slightly altered sequence to the previous report (B.W. Gibson, W.C. Herlihy, T.S.A. Samy, K-S, Hahn, H. Maeda, J. Meienhofer and K. Biemann, *J. Biol. Chem.* **1984**, *259*, 10801. A prosthetic group in NCS has been reported (42-44), of which chemical nature consists of naphthalene carboxylic acid, diacyl peroxide and fucosamine (Edo, K., personal communication), with an estimated mass of 661. Thus, a new molecular weight of NCS is 11,741, in stead of 10,717 is used in this study.
(5) (a) Takeshita, J.; Maeda, H.; Koike, K. *J. Biochem. (Tokyo)* **1980**, *88*, 1071. (b) Maeda, H.; Kumagai, K.; Ishida, N. *J. Antibiot.* **1966**, *19*, 253.
(6) Maeda, H.; Glaser, C. B.; Kuromizu, K.; Meienhofer, J. *Arch. Biochem. Biophys.* **1974**, *164*, 379.
(7) Maeda, H. *J. Antibiot.* **1974**, *27*, 303.
(8) Maeda, H.; Ichimura, M.; Sato, H.; Ohtsuki, K. *J. Antibiot.* **1978**, *31*, 468.
(9) Takeshita, J.; Maeda, H.; Kanamaru, R. *Gann* **1982**, *73*, 278.

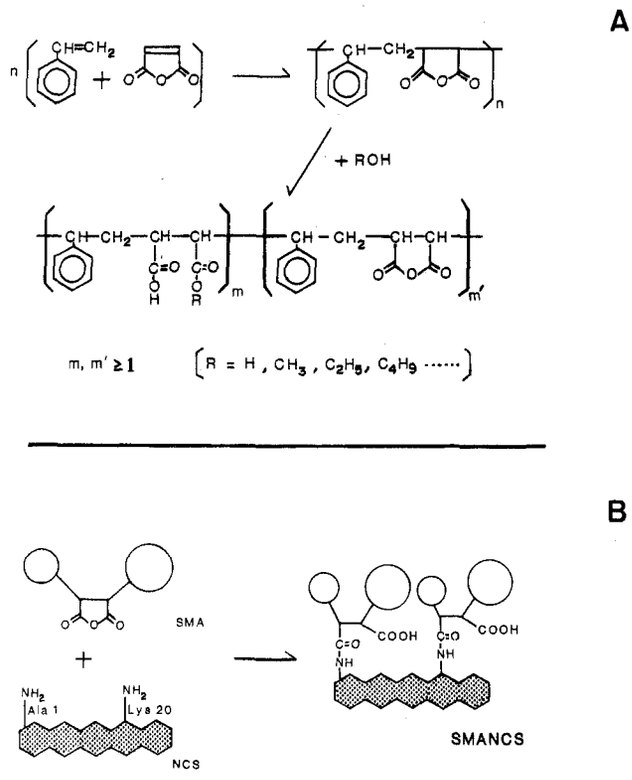
0022-2623/85/1828-0455$01.50/0

Table I. Analysis and Solubility of p-E-SMA

type of SMA	M_w	M_w/M_n	M_{An},[d]	solubility					
				n-hexane	toluene	acetone	dioxane	H_2O[e]	0.5 M $NaHCO_3$
p-H-SMA[a]	1760	1.20	48.3	insol	swell	soluble	soluble	insol	soluble
p-Bu-SMA[b]	2270	1.17	30.0	insol	swell	soluble	soluble	insol	soluble
p-EtCe-SMA[c]	1700	1.18	25.3	insol	swell	soluble	soluble	insol	soluble

[a] Partially hydrolyzed SMA. [b] Partially half-butyl-esterified SMA. [c] Partially half-ethylcellosolve-esterified SMA. [d] Residual content of maleic anhydride residue. [e] pH 5.0.

Scheme I



ticularly in the tumor[10] has recently been found. Furthermore, a preliminary experiment showed that the strand break of DNA in vitro by SMANCS was observed,[9] and unpublished observation revealed that it causes chromosomal breakage predominantly in the tumor cells as revealed by the analysis by fluorescence-activated cell sorter (Maeda et al., unpublished). In addition to these facts, NCS appears to be regenerated from SMANCS when mixed with tissue homogenates,[9] exhibiting a character of a prodrug of NCS. Therefore, the present method appears to be generally applicable for the improvement of the in vivo character of biologically active proteins while maintaining the parental activity at the molecular level.

Similar efforts along this line, that is, macromolecular therapeutics, have recently been published: low-molecular-weight anticancer agents such as daunorubicin and methotrexate were coupled to a number of polypeptides or proteins. The pharmacological improvements in slow release, lower toxicity, and pronounced antitumor activity were found to be remarkable.[11-14]

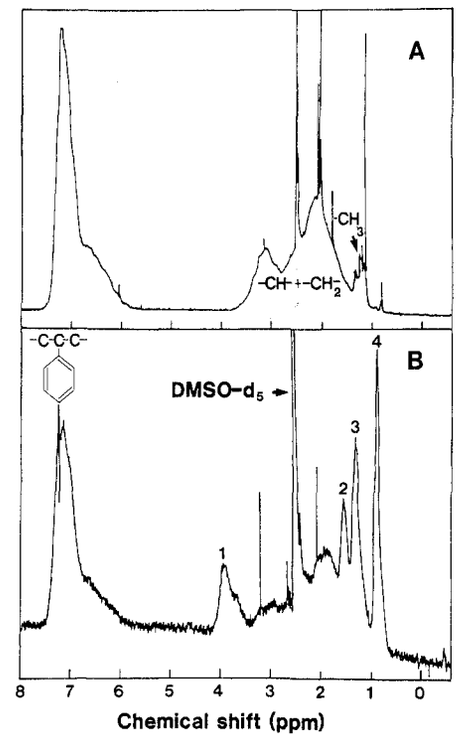


Figure 1. ^1H NMR (500 MHz) spectra of the fractionated (A) and its partially half-butyl-esterified SMA (B). Samples were dissolved at 5% in dimethyl-d_5 sulfoxide (Me_2SO-d_5) at 40 °C. A few droplets of trifluoroacetic acid was added so that the peak due to H_2O can be shifted outside the region shown in the figure. The peaks indicated by the numbers 1–4 in B are the signals of proton located in the corresponding positions of carbon in the butyl group. A broad bimodal peak appearing from 1.4 to 3.7 ppm in A is due to the methylene and tertiary carbon of the main chain. The peak due to the carboxyl group which appears at 12 ppm was not detected for SMA while it appeared for p-Bu-SMA (not shown).

Results

The overall reaction scheme is shown in Schemes IA and IB. To clarify the nature of the SMA polymer obtained, ^1H and ^{13}C NMR studies were undertaken. The broad signals from 1.4 to 3.7 ppm and the neighborhood of the ^1H NMR spectra (Figures 1A and 1B) show signals of CH and CH_2 of the main chain. The methyl group near 1.25 ppm is the signal from the terminal cumene residue in the SMA chain. The signal near 7.1 ppm indicates a proton signal of the styrene residue. Computation of the ratio of styrene and maleyl residues in the proton peak area indicated a character of 1:1 copolymer. The degree of polymerization of styrene–maleyl was found to be 7–8 with use of the methyl group of cumene as one unit, and $(M_n)_s$ data showed a good agreement. Sharp spikes between 0.8 and 3.2 ppm reflect signals from Me_2SO and acetone and *n*-hexane used for fractionation. The ^{13}C NMR data (not shown) showed concordant results to those of ^1H NMR and

(10) Maeda, H.; Iwai, K.; Morinaga, T.; Konno, T. *Igaku no Ayumi* **1983**, *124*, 25 (in Japanese).
(11) Carraher, C. E., Jr.; Gebelein, C. G., Ed. "Biological Activities of Polymers"; American Chemical Society: Washington, DC, 1982; ACS Symp. Series No. 186.
(12) Ryser, H. J-P.; Shen, W.-C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3867.
(13) Trouet, A.; Masquelier, M.; Baurain, R.; Depreze-De Compeneere, D. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 626.
(14) Zunino, F.; Gambetta, R.; Vigevani, A.; Penco, S.; Geroni, C.; DiMarco, A. *Tumori* **1981**, *67*, 521.

Table II. Synthesis, Analysis, and Solubility of SMANCS

reaction			chemical Analysis					solubility[e]	
type of SMA[a] in SMANCS	feed ratio[b] (w/w)	time,[c] h	NH_2 group modified, mol %	N, %	C, %	H, %	P,[d] wt %	H_2O (pH 5)	5 mM HN_4HCO_3
p-H-SMA	4.5	24 (7.5)	97.3	10.02	52.28	6.18	68.8	insol	soluble
p-Bu-SMA	7.3	24 (14)	91.2	9.85	54.28	6.29	63.6	insol	soluble
p-EtCe-SMA	5.5	24 (10)	93.0	10.47	52.94	6.25	55.6	insol	soluble

[a] See footnote in Table I. [b] Total weight of p-E-SMA divided by the weight of NCS. [c] Values in the parentheses: the time required to dissolve p-E-SMA. [d] Protein content; determined by the method of Lowry (1951). [e] Solubility was examined by adding 1 mg of SMANCS to 1 mL of the solvent.

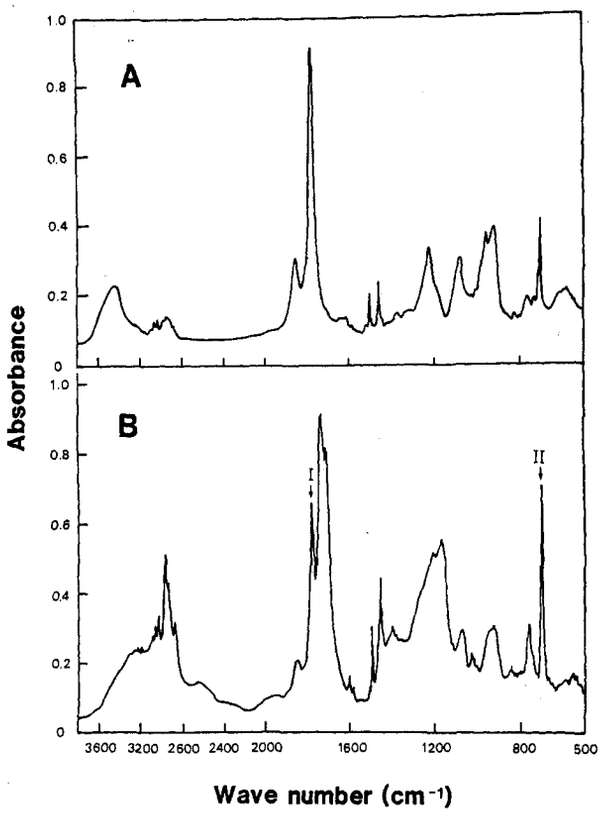


Figure 2. Infrared spectra of a fractionated SMA (A) and its partially half-butyl- esterified product (B). The peaks I (stretching of carbonyl group of anhydride) and II (stretching of C–H of aromatic group of styrene) were used for the quantitation of half-butylation.

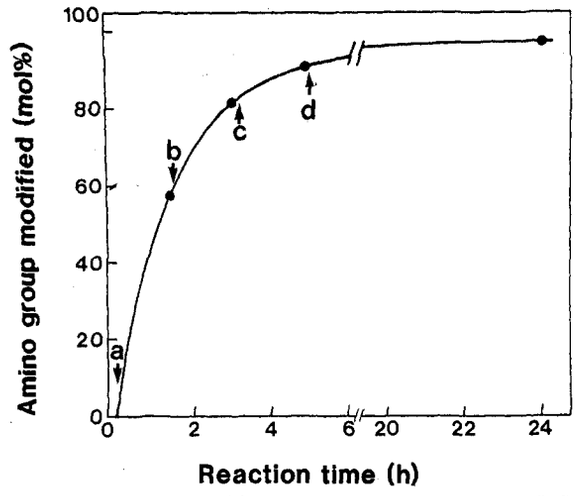


Figure 3. Reaction of NCS with partially hydrolyzed (p-H)-SMA. To a solution of NCS (0.2 g) in 0.8 M $NaHCO_3$ (5 mL) at 4 °C in the dark was added powdery p-H-SMA at the times indicated by the arrows (a, 0.3 g; b, 0.3 g; c, 0.2 g; d, 0.1 g). The reaction mixture was stored in a refrigerator after the complete dissolution of the last addition (d). Twenty-four hours after the incipience of the reaction, the amino group modified was 97.3 mol %.

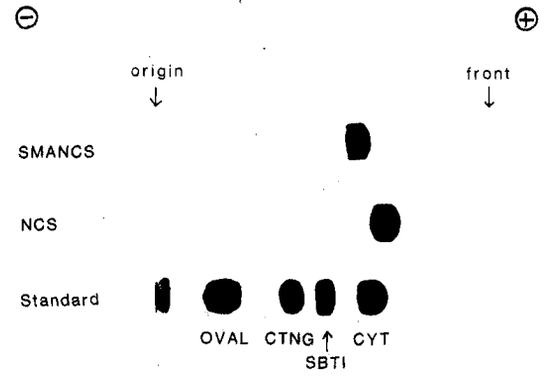


Figure 6. Disc gel electrophoresis in the presence of $NaDodSO_4$ at 0.2% in 0.025 M Tris-glycine buffer containing 2 mM EDTA, pH 8.3. Standard proteins and their molecular weight (in parentheses) are as follows: OVAL, ovalbumin (48 000); CTNG, chymotrypsinogen (24 000); SBTI, soybean trypsin inhibitor (20 000); CYT, cytochrome *c* (14 000); NCS, neocarzinostatin (12 000).

that of Buchuk and Ramey.[15] IR spectra of SMA before and after the partial half-*n*-butylation (p-Bu-SMA) are shown in Figures 2A and 2B. The representative p-E-SMA preparations contained about 2 mol of anhydride residues/mol of SMA. Analyses and some physicochemical properties are listed in Table I.

In Figure 3, the reaction of the amino group of NCS is plotted as a function of reaction time. In the reaction a 4.5 times excess of p-H-SMA over NCS by weight (22 molar excess of SMA/amino group) was used at about pH 8.5. Within these experimental conditions almost complete reaction of the amino group of NCS was observed, whereas the reactions of both p-Bu-SMA and *p*-ethylcellosolve (EtCe)–SMA with NCS were found to be appreciably slow.

Figure 4 shows a representative gel filtration profile for the purification of SMANCS on a Sephadex G-75 column (see paragraph concerning supplementary material). A fair separation was achieved. Analysis by HPLC as shown in Figure 5 (supplementary material) indicates a single peak of SMANCS without contamination of p-H-SMA after the Sephadex G-75 chromatography. Gel electrophoresis with $NaDodSO_4$ was applied to assess the purity and to estimate the molecular weight of the purified SMANCS (Figure 6). The results showed the apparent molecular weight of biantennary SMANCS to be 17 000 (Table III). The solubility and other properties of purified SMANCS are listed in Table II.

The $\bar{M}_w$ of SMANCS was estimated from either nitrogen content by elementary analysis or protein content by using the following equations:

$$\bar{M}_w = 11\,741 \times (14.24/N) \quad (1)$$

from nitrogen content (N) and

$$\bar{M}_w = 11\,741 \times (100/P) \quad (2)$$

(15) Buchuk, B. E.; Ramey, K. C. *J. Polym. Sci., Polym. Lett. Ed.* **1976**, *14*, 401.

Table III. Molecular Weights of SMANCS

SMANCS	molecular weight estimation based on						
			HPLC				
	nitrogen content	protein content	SMA[a]	protein[b]	$NaDodSO_4$-PAGE	FP[c]	theor[d]
H-SMANCS	16455	17113			17000		15261
Bu-SMANCS	17003	18430	12200	30000	17000	17000	16281
EtCe-SMANCS	16162	21062			17000		15101

[a] Using SMA as standard. [b] Using protein as standard. [c] Fluorescence polarization (Figure 8 in the text). [d] See Appendix. Note that parental neocarzinostatin has a molecular weight of 11741. (See ref 4.)

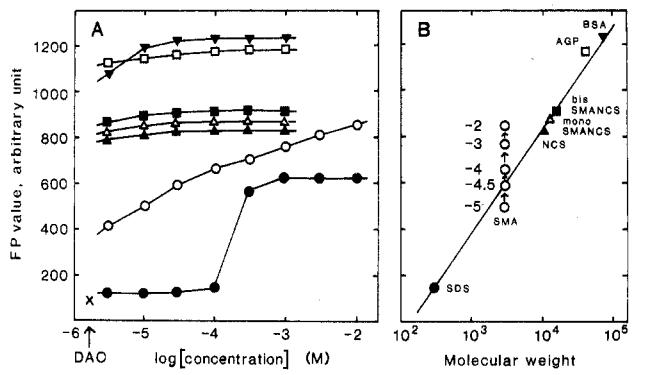


Figure 8. (A) Fluorescence polarization (FP) values of noncovalently bound dodecyl acridine orange on various compounds expressed in arbitrary unit at 25.0 °C. An arrow on the bottom (DAO) indicates the concentration of dodecyl acridine orange used in the cuvette for labeling. Symbols for each substance and respective molecular weight in parentheses are as follows: (▼) BSA, bovine serum albumin (68000), (□) AGP, human α_1-acid glycoprotein (40000), (■) bisSMANCS, biantennary SMANCS (17000), (△) monoSMANCS, monoantennary SMANCS (14300), (▲) NCS (12000), (○) SMA, *n*-Bu-SMA (2700), (●) $NaDodSO_4$ sodium dodecyl sulfate (290), and (×) DAO, dodecyl acridine orange (460). (B) Observed relationship between FP values in arbitrary units and molecular weight. The same symbols are used in A and B. The numbers by the open circles in B indicate concentrations (M) of SMA in log unit.

from protein content (*P*, wt %), where 11741 and 14.24 are the molecular weight and the nitrogen content of NCS, respectively.[6] The molecular weight data shown in Table III are in good agreement with each other and with those obtained by theoretical calculations. See the Appendix for theoretical justification of the procedure (supplementary material).

Apparent molecular weights from the partition coefficient K_d, based on known molecular weights of SMA (Figure 7, supplementary material), polyacrylamide gel electrophoresis with $NaDodSO_4$ (Figure 6), and fluorescence polarization (Figure 8), are also listed in Table III. All three values are in good agreement with those obtained by using eq 1 and 2 (16000–18000). Data obtained by HPLC yielded different values when proteins of various molecular weights were used as the standard for calibration. The spectroscopy in the near-UV region showed that the so-called nonprotein chromophore was retained in SMANCS (data not shown; see ref 4).

In Tables IV and V, the biological activities of SMANCS are summarized. The biological half-life of SMANCS in the serum and blood has been prolonged; the best results were obtained for Bu-SMANCS. Antibacterial activity was not altered significantly on a molar basis, and the antitumor activity in mice bearing ascitic S-180 cells has been improved to a great extent (Table V), but no appreciable antitumor activity was observed for SMA or SMA–BSA. The acute toxicity, as expressed by the LD_{50}, decreased to about one-fourth that of NCS. Thus, the properties of parental NCS in vivo have been greatly improved by the polymer conjugation.

Table IV. Biological Activity of Neocarzinostatin, Various SMA, and Its Polymer Conjugate (SMANCS)

	in activation half-time,[a] min		antibacterial activ,[b] %	LD_{50}[c]	
	serum	blood		ip[d]	iv[e]
n-Bu-SMA			0[f]	133	100
Et-SMA			0	59.6	50
H-SMA			0	11.7	
Bu-SMANCS	19	30	70	4.6	3.4
Et-SMANCS	6	8	60	3.7	2.3
NCS	2	3	100	1.3	1.0

[a] 100 μg/mL of each drug was incubated at 37 °C in blood or serum and an aliquot was taken out at each time interval. Values of remaining antibiotic activity in log unit determined by a standard curve were plotted against the incubation time and the residual activity required for 50% are shown. [b] 100 μg/mL of drugs were assayed on an agar plate by growth inhibition of *Sarcina lutea* (see text). [c] Based on the Litchfield–Wilcoxon method. LD_{50} in milligrams/kilogram. [d] One intraperitoneal injection (milligrams/kilogram) in mice 5–6 weeks old with body weights of 22–28 g. [e] One intravenous tail injection (milligrams/kilogram) in rats 5–6 weeks old with body weights of 130–170 g. [f] Value was zero even tested at 10000 g/- ᵀᵤ.

Table V. Antitumor Activity of SMANCS and NCS in Mice Bearing S-180 Ascitic Tumor Cells

group[a] and drug	drug dose,[b] mg/kg	mean survival, days	increase in life span, %	no. of mice survived on day 50
control, no drug	0	12.3 ± 2.9	0	0
Bu-SMA	0.1	13.6 ± 4.9	10	0
	0.25	14.5 ± 4.2	17	0
	0.5	11.5 ± 3.7	−6	0
	1.0	12.4 ± 2.0	0.8	0
SMA–BSA[c]	0.1	11.4 ± 3.4	−7	0
	0.25	15.1 ± 3.5	22	0
	0.5	14.3 ± 4.8	16	0
	1.0	15.1 ± 4.0	22.7	0
Bu-SMANCS	0.25	>32.0 ± 9.4	>163	2
	0.5	>36.6 ± 9.4	>198	2
	1.0[c]	>40.2 ± 8.0	>227	4
NCS[d]	0.01[e]	27.5 ± 12.6	127.2	0
NCS + Bu-SMA	0.1/0.01	28.2 ± 12.8	>133.1	1
(Bu-SMA/NCS)	0.25/0.01	28.7 ± 9.7	137.2	0

[a] Tumor inoculation; 1 × 10^6 cell intraperitoneally per mouse. Ten mice per group. [b] This amount was administered ip once daily on 5 successive days. Treatment started 24 h after tumor inoculation. [c] Conjugate of Bu-SMA with bovine serum albumin. [d] Twenty mice per group. [e] Maximum tolerable dose (no toxic death) under this protocol.

Discussion

We have previously found that complete modification of all the amino groups of NCS, one at the N-terminus and the other at lysine-20, with succinic anhydride did not affect the molecular function of NCS.[7,8] That is, the effect of modified NCS on strand breaks of DNA and inhibition of DNA synthesis remained unaffected, whereas its stability in serum and antitumor activity were improved considerably.[8,16] However, the succinylation did not alter

(16) Maeda, H.; Takeshita, J. *J. Antibiot.* **1976**, *29*, 111.

the properties of NCS in vivo enough to make it a sufficiently different and more ideal antitumor agent.

The primary requisite for an ideal antitumor agent is efficient accumulation in the tumor tissue and the lymphatics. The latter is crucial for antimetastatic activity. It is known in lymphology that when high-molecular-weight substances leak out of the blood capillaries, they are recovered only through the lymphatic system.[17] This means that such substances, small enough to leak out of the capillaries but large enough to be prevented from backward diffusion into the blood capillaries, would be recovered in the lymph. This notion was partially verified in vivo for NCS,[18] which is about 12000 daltons. On the basis of this information, we attached SMA derivatives to NCS to increase its molecular weight and lipophilicity. A preliminary account of this has been reported.[19,20] Subsequent studies of the distribution of SMANCS in vivo supported the above assumption.[9] The purpose of the present experiments was to elaborate the chemistry of SMANCS in more detail.

The reaction of the amino group of NCS with p-H-SMA was about 97% complete within 24 h (Figure 3). This indicates that almost all the free amino groups of NCS were modified. Although there might be a possible reaction with hydroxyl groups, circumstantial evidence has so far proved that no such reaction product was formed, notwithstanding a possible hydrolysis of SMA linked through ester linkage during purification. This result agrees with those of the elemental analysis and the molecular weight estimation as described above. The size of the alkyl group (R in Scheme IA) did not affect the reaction rate greatly (Table II).

Some difficulties were encountered in the separation of SMANCS from the reaction mixture when ordinary methods such as fractional precipitation, ion-exchange chromatography, and adsorption chromatography were employed. However, separation by dialysis followed by gel filtration was found to be satisfactory within the present experimental conditions (Figures 5 and 6).

The purified SMANCS, in which two amino groups were modified (biantennary), exhibited homogeneity upon HPLC (Figure 5A) and gel electrophoresis with $NaDodSO_4$ (Figure 6). Physical and chemical properties of SMANCS were considerably different from the parental NCS (Tables II and III). Major differences between SMANCS and NCS involved their solubility profiles and molecular weights. SMANCS became soluble in aqueous alkaline pH and in some organic solvents (not shown). This increase in lipophilicity made SMANCS highly lymphotropic[9] and antimetastatic.[20] This unique solubility made it possible to dissolve SMANCS in a lipid contrast medium, Lipiodol (Andre-Guelbet, Paris, France). As a consequence, highly selective drug targeting as well as tumor imaging under X-ray became possible.[21-23] The biantennary SMANCS had a molecular weight of about 15500 as determined by nitrogen content and about 17000 by protein content, disc gel electrophoresis with $NaDodSO_4$, FP method (Table III, Figure 8), and the HPLC method using SMA as the molecular standard (Figure 7). All values are in good agreement (Table III). It is interesting, however, to note that the molecular weight of SMANCS obtained by HPLC on a TSK G-3000 SW column with various proteins as calibration standards appeared much higher than that with SMA as standard. This discrepancy may be explained as follows. At a fixed degree of polymerization, the molecular volume of a given linear flexible polymer is known to be larger than that of a globular protein in dilute solution.[24]

We have also examined the molecular volume by FP with dodecyl acridine orange as fluorescent probe, which is supposed to bind noncovalently to both hydrophobic and anionic regions.[25] Various standard proteins exhibited a linear relationship between the arbitrary FP value, which reflects the molecular volume in the Perrin equation, and the log of molecular weight (Figure 8B). Mono- and biantennary SMANCS showed higher molecular volumes than NCS, which corresponded to their molecular weights. It is noteworthy, however, that under these experimental conditions p-Bu-SMA revealed a monotonic increment of the FP value parallel to its concentration, and it reached its effective molecular volume close to that of biantennary SMANCS, at above 10^{-2} M. This result may be interpreted to mean that the intermolecular association of SMA increases in a concentration-dependent manner (Figure 8A). Therefore, a reduction of the concentration of SMA by dialysis prior to gel filtration is recommended to facilitate the complete removal of SMA from the reaction mixture.

As a consequence of the present polymer conjugation, it became possible to alter in vivo properties of NCS greatly without serious difficulty. Furthermore, some of the shortcomings, such as short half-life in vivo, could be overcome, and tumor targeting was achieved.[10,21-23] Another shortcoming of proteinaceous drugs, namely, antigenicity, may also be overcome by the attachment of polymers. For instance, the antigenicity of L-asparaginase and uricase decreased greatly by conjugation of poly(ethylene glycol), but their original activity at the molecular level was retained.[26,27] A recent article by Ulmer[28] described the engineering of protein molecules to improve enzyme properties.

Although undescribed in the present report, the biological activity of polyanion such as induction of interferons and interleukins and activation of macrophages was evisaged. In a separate experiment, we have found a stimulation of peritoneal macrophage by SMANCS and other SMA-conjugated proteins.[29]

Therefore, the antitumor activity of SMANCS may depend on the direct action of the NCS component in it, but it may also be ascribed to the SMA component to some extent.

The present results provide another example of protein engineering in an attempt to fulfill and improve the bio-

(17) Courtice, F. C. "Lymph and the Lymphatic System"; Mayersen, H. S., Ed.; C. C. Thomas Co., Springfield, IL, 1963; pp 89-126.
(18) Maeda, H.; Takeshita, J.; Yamashita, A. *Eur. J. Cancer* **1980**, *16*, 723.
(19) Maeda, H.; Takeshita, J.; Kanamaru, R. *Int. J. Peptide Protein Res.* **1979**, *14*, 81.
(20) Maeda, H.; Takeshita, J.; Kanamaru, R.; Sato, H.; Khatoh, J.; Sato, H. *Gann* **1979**, *70*, 601.
(21) Konno, T.; Maeda, H.; Iwai, K.; Tashiro, S.; Maki, S.; Morinaga, T.; Mochinaga, M.; Hiraoka, T.; Yokoyama, I. *Eur. J. Cancer Clin. Oncol.* **1983**, *19*, 1053.
(22) Konno, T.; Maeda, H.; Iwai, K.; Maki, S.; Tashitro, S. *Cancer* **1984**, *54*, 2367.
(23) Iwai, K.; Maeda, H.; Konno, T. *Cancer Res.* **1984**, *44*, 2115.
(24) Tanford, C. *Adv. Protein Chem.* **1968**, *23*, 121.
(25) Kubota, Y.; Kodama, M.; Miura, M. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 100.
(26) Ashihara, Y.; Kono, T.; Yamazaki, S.; Inada, Y. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 385.
(27) Nishimura, H.; Matsushima, A.; Inada, Y. *Enzyme* **1981**, *26*, 49.
(28) Ulmer, K. M. *Science* **1983**, *219*, 666.
(29) Oda, T.; Maeda, H. Proceedings of the Japanese Cancer Association, 42nd Annual Meeting, October, 1983, Nagoya; Abstr 939.

logical and pharmacological objectives of macromolecular drugs in vivo.

Experimental Procedures

Materials. NCS was obtained from Kayaku Antibiotics Research Laboratories Co. Ltd. (Tokyo, Japan) and used without further purification. Various derivatives of SMA were obtained as described below. Either HPLC grade or special grade solvents were supplied from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan), and they were dried over 4A molecular sieves of HPLC grade (E. Merck, Darmstadt, West Germany) and filtered immediately before use. Dodecyl acridine orange was a product of Dojin Chemical Research Laboratories Inc. (Kumamoto, Japan) and was found to be pure by thin-layer chromatography. All other chemicals were special grade and were obtained from commercial sources unless otherwise stated.

Copolymerization of Styrene and Maleic Anhydride.[30] The copolymerization of styrene and maleic anhydride was carried out in cumene at its boiling point (152–153 °C) by using dicumyl peroxide as the initiator[30] (Scheme IA). The copolymer was precipitated at room temperature by adding *n*-hexane. The number-average molecular weight ($\bar{M}_n$) of SMA used in the following experiment was measured by vapor pressure osmometer (VPO) as described below and was found to be 1640.

Fractionation of SMA. A column elution method[31,32] was applied for the fractionation of the SMA obtained above with use of mixtures of acetone and *n*-hexane at 25 °C. SMA was deposited on the surface of glass beads (mean particle diameter of 0.1 mm), which were then packed in a glass column. The solvent mixtures of acetone and *n*-hexane were used for elution, with acetone concentration increasing in a stepwise manner. An appropriate fraction thus obtained was used in the following experiments.

Chemical Derivatization of Fractionated SMA. Both partial half-esterification and partial hydrolysis of SMA were carried out in dioxane at 90 °C with use of lithium acetate as a catalyst.[33] After the reaction, the sample was lyophilized and then dried in vacuo at 90 °C. The anhydride content was determined on the basis of relative absorption at 1780 and 700 cm^{-1}. Either partially esterified (p-E-) or partially hydrolyzed (p-H-) SMA was employed for the reaction with NCS.

Chemical Coupling of p-E- or p-H-SMA to NCS. A typical coupling reaction of NCS with p-E- or p-H-SMA, as shown diagrammatically in Scheme IB, was carried out at 4 °C in the dark by stepwise addition of the SMA derivative to the solution of NCS in 0.8 M $NaHCO_3$ while the solution was stirred. The reaction of the amino group of NCS was quantified by the TNBS method.[34]

Purification. Removal of p-E- or p-H-SMA by precipitation from the reaction mixture by lowering the pH was unsuccessful due to the insolubility of all the components below pH 7.0. Cation-exchange chromatography such as CM-cellulose was inapplicable due to insolubility in the acidic medium. DEAE-cellulose column chromatography showed little separation of the conjugates (SMANCS) because of the anionic similarity of each component. Precipitation with either ammonium sulfate nor sodium sulfate proved unsuccessful.

A satisfactory result was obtained by dialysis in a mild alkaline pH followed by gel chromatography. Namely, the reaction mixture was dialyzed in a Visking tube (VT-803, Union Carbide Corp., Chicago, IL) against 10 mM NH_4HCO_3. Subsequently, the dialysate was purified by gel chromatography on a Sephadex G-75 column with 10 mM NH_4HCO_3 as a carrier solvent. The eluate was monitored at 254 nm. The peak containing SMANCS was lyophilized.

Vapor Pressure Osmometry. A vapor pressure osmometer (VPO) (Kanauer GmbH, Berlin, West Germany), was operated at 45 °C with ethyl acetate as solvent for SMA and its derivatives. The equipment was calibrated by using recrystallized benzil as usual. The measurements and analyses of the data obtained were performed according to the instructions of the manufacturer to obtain $\bar{M}_n$ values.

High-Pressure Liquid Chromatography. HPLC of SMA and its derivatives was carried out with a Model 150C ALC/GPC (Waters Inc., Bedford, MA) at 24 °C in combination with a Shodex A-803 column (Showa Denko Co., Ltd., Tokyo, Japan). The elution was performed with tetrahydrofuran as the carrier solvent at a flow rate of 1 mL min^{-1}. Molecular weights of fractionated SMA, p-E-SMA, and p-H-SMA were determined by VPO. Molecular weight distribution (MWD) and $(\bar{M}_w)_s$ and $(\bar{M}_n)_s$ were calculated by means of a Waters Data Module.

HPLC of crude and purified SMANCS as well as NCS was carried out with Model 244 ALC/GPC (Waters Inc.) and a TSK G-3000SW column (Toyo Soda Co., Ltd., Tokyo, Japan). Elution was performed at 1 mL min^{-1} at pH 7.9 with 10 mM NH_4HCO_3 containing 30 mM NaCl as the carrier solvent. The eluate was monitored at 254 and 280 nm.

Nuclear Magnetic Resonance. ^1H NMR (500 MHz) and 125-MHz ^{13}C NMR spectra of the solutions of SMA and its derivatives in dimethyl-d_5 sulfoxide were obtained at 40 °C by using a JEOL Model GX-500 NMR instrument (Tokyo, Japan).

Infrared Spectrometry. Absorption spectra of SMA and its derivatives were obtained by using a Digilab Model FTS-20C spectrometer (Digilab Inc., Cambridge, MA) and the KBr tablet method.

Protein Content. Protein content in SMANCS was determined by the method of Lowry et al.[35] NCS was used as the calibration standard.

$NaDodSO_4$ Gel Electrophoresis on Polyacrylamide Gel. Disc gel electrophoresis was carried out in 12% acrylamide in 0.03 M Tris-glycine buffer (pH 8.0) containing 2 mM EDTA and 0.2% $NaDodSO_4$ according to the method of Weber and Osborn.[36] Approximately 10 μg each of the standard proteins and 20 μg of SMANCS were electrophoresed. They were stained with Coomassie Blue R-250 and destained with aqueous acetic acid.

Fluorescence Polarization. Measurements were carried out in 0.05 M Tris-HCl buffer (pH 8.0) by using Model MAC-II fluorescence spectropolarimeter of Japan Immunoresearch Laboratories, Ltd. (Takasaki, Japan), under strict temperature control at 25.00 ± 0.05 °C. Dodecyl acridine orange was used to label proteins and other derivatives noncovalently. Excitation and emission wavelengths were 490 and 520 nm, respectively. The polarization values were printed automatically. The relative molecular volume of the solution was obtained by analyzing the data according to the Perrin equation.[37]

Biological Activity Assay. Bioassays of NCS and SMANCS were performed as described previously by using the Gram-positive bacterium *Sarcina lutea* on an agar plate of diluted Müller–Hinton medium,[18] which is based on growth inhibition. Heparinized human blood or serum used for drug inactivation without addition of protease inhibitors was obtained from a volunteer.

Antitumor Activity and Toxicity. The assays were carried out by using ICR-JCL strain male mice 6–7 weeks old with an average body weight of 24–30 g. Ten mice were used per group. A 0.5-mL sample of S-180 ascitic tumor cell suspension containing 1×10^6 cells was injected intraperitoneally into each mouse for the antitumor assay. Twenty-four hours after tumor inoculation, the drugs were injected intraperitoneally, after which they were

(30) Muskat, I. E. U.S. Patent 3 085 994, 1963.
(31) Ueda, M. *Polymer J.* **1972**, *3*, 431.
(32) Barral II, E. M.; Johnson, J. F.; Cooper, A. R. "Fractionation of Synthetic Plymers"; Tung, L. H., Ed.; Marcel Dekker: New York, 1977; pp 267–344.
(33) Muskat, I. E. U.S. Patent 3 392 155, 1968.
(34) Fields, R. *Methods Enzymol.* **1972**, *25*, 464.
(35) Lowry, O. H.; Rosenborough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
(36) Weber, K.; Osborn, M. *J. Biol. Chem.* **1969**, *244*, 4406.
(37) Maeda, H. *Anal. Biochem.* **1979**, *92*, 222.
(38) Schulz, G. V. *Z. Phys. Chem.* **1939**, *B43*, 25.
(39) Zimm, B. H. *J. Chem. Phys.* **1948**, *16*, 105.
(40) Kotlair, A. M. *J. Polym. Sci.* **1964**, *A2*, 1373.
(41) Feller, W. "Introduction to Probability Theory and Its Applications", 1st ed.; Wiley: New York, 1967; Vol. 2, pp 45–47.
(42) Albers-Schönberg, G.; Dewey, R. S.; Hansen, O. D.; Liesch, J. M.; Napier, M. A.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1351.
(43) Koide, Y.; Ishii, F.; Hasuda, K.; Koyama, Y.; Edo, K.; Katamine, S.; Kitame, F.; Ishida, N. *J. Antibiot.* **1980**, *33*, 342.
(44) Edo, K.; Katamine, S.; Kitame, F.; Ishida, N.; Koide, Y.; Kusano, G.; Nozoe, S. *J. Antibiot.* **1980**, *33*, 347.

injected once daily for 5 successive days. Bu-SMA described in Table I was used in the present experiments after reacting with glycine; thus little anhydride residue would remain intact. All other tested materials were used without any special modifications. The survival rate, expressed as the increase in life span over nontreated controls, was calculated 50 days after drug treatment. Male Donryu rats weighing about 250–300 g were also used for the toxicity (LD_{50}) assay.

Acknowledgment. We gratefully acknowledge the excellent cooperation of our colleagues Ryuzo Asano, Osamu Nakaji, Koichiro Kawai, Toshihiko Yoshitake, and Isao Akuta of Kuraray Co., Ltd.. Thanks are also due to Dr. Yoshiharu Amiya for NMR and to Yasuyo Omori for infrared spectroscopy and to Professor K. Kawahara of Nagasaki University for the gift of purified pullulan. This work was supported in part by an Award from the Japanese Foundation for Multidisciplinary Treatment of Cancer (1982) and a Cancer Research Grant from the Ministry of Education, Science and Culture of Japan (to H.M. for 1983 and 1984).

Supplementary Material Available: Figures 4, 5, and 7 and an appendix for theoretical justification for the molecular weight estimation (5 pages). Ordering information is given on any current masthead page.

Analgesics of the 6,14-Ethenomorphinan Type. 6-Deoxy-7α-orvinols and 6-Deoxy-8α-orvinols

Laura L. Knipmeyer and Henry Rapoport*

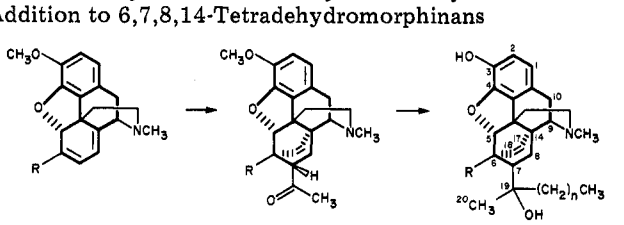
Department of Chemistry, University of California, Berkeley, California 94720. *Received June 11, 1984*

6-Deoxythebaine (3) has been prepared as a precursor to C-6 alkyl substituted orvinols 15 and 17. The C-6 methyl group was introduced by addition of methyllithium to codeinone. Transformation of 6-methylcodeine to its 6-methyl ether and 1,4-elimination of methanol with potassium *tert*-butoxide in Me_2SO then gave 6-deoxythebaine (3) in 49% overall yield. Diels–Alder addition of methyl vinyl ketone to this diene yielded four ketones: three regio- and stereoisomeric 6,14-*endo*-ethenomorphinans and one exo adduct. The major ketone isomer provided the set of C-19 diastereomeric orvinols 15 in which the pendant carbon has the 7α configuration. Regioisomeric ketone 8, in which the acetyl group is at C-8, was formed in 3% yield and was similarly converted to the corresponding orvinols 17. Orvinol (*R*)-15 (*R* at C-19) is an analgesic of very high potency, 2200 times that of morphine; regioisomeric orvinols 17, in which the pendant tertiary alcohols are on C-8, are much less potent. The higher activity of the C-6 methyl and methoxyl analogues (*R*)-15 and (*R*)-22 relative to hydrogen-substituted (*R*)-19 indicates that C-6 alkyl substitution enhances analgesic potency.

Alkylorvinols (18, 22) are extremely potent bicyclic analogues of morphine. For example, etorphine[1] (19-(*R*)-propylorvinol, 18), used for immobilization of large animals for game conservation and veterinary purposes, has an analgesic potency 1000 times that of morphine.[2] The bicyclic structure of the orvinols is formed by Diels–Alder addition of dienophiles to thebaine (1).[3] Thus methyl vinyl ketone addition gives thevinone (11), and addition of organometallic reagents to the ketone[4] at C-19 affords diastereomers that vary greatly in activity depending on the absolute stereochemistry at C-19. *R* diastereomers are highly potent, while *S* isomers have activity in the range of morphine. Hydrogen bonding between the C-6 oxygen and the C-19 hydroxyl resulting in conformational stabilization was proposed[5] as an explanation for the large difference in activity dependent on C-19 stereochemistry. The subsequent synthesis and pharmacological evaluation of 6-demethoxyorvinols (19)[6] revealed that the relationship between high activity and C-19 stereochemistry is independent of intramolecular hydrogen bonding (Scheme I).

We therefore considered synthesis of 6-alkyl analogues of the orvinols 22 for investigation of structure–activity relationships based on the C-6 substituent. For this purpose, the synthesis of 6-deoxythebaine (3) is required as substrate for the analogous Diels–Alder addition of methyl vinyl ketone to give bicyclic 6-deoxythevinone (7). Also, in the absence of the polarizing methoxy functionality at C-6, Diels–Alder addition to diene 3 can give a regioisomeric adduct in which the acetyl group is attached at C-8. Ketone 8 therefore was sought as the precursor to orvinol analogues having the stereochemically important pendant C-19 center on the adjacent C-8 carbon atom.

Scheme I. Synthesis of 19-Alkylorvinols by Diels–Alder Addition to 6,7,8,14-Tetradehydromorphinans



Chemistry. Synthesis of 6-deoxythebaine (3) was accomplished by first introducing the C-6 methyl group by methyllithium addition to codeinone, followed by 1,4-elimination from the resulting 6-methylcodeine (4). Although methyllithium addition to codeinone in ether at 0 °C has been reported[7] to give a high yield, we found that reaction in THF at −78 °C was more reproducible and

(1) Bentley, K. W.; Hardy, D. G. *J. Am. Chem. Soc.* **1967**, *89*, 3281.
(2) Lewis, J. W.; Bentley, K. W.; Cowan, A. *Ann. Rev. Pharmacol.* **1971**, *11*, 241.
(3) Bentley, K. W.; Hardy, D. G. *J. Am. Chem. Soc.* **1967**, *89*, 3267.
(4) Bentley, K. W.; Hardy, D. G.; Meek, B. *J. Am. Chem. Soc.* **1967**, *89*, 3273.
(5) Loew, G. H.; Berkowitz, D. S. *J. Med. Chem.* **1979**, *22*, 603.
(6) Hutchins, C. W.; Cooper, G. K.; Purro, S.; Rapoport, H. *J. Med. Chem.* **1981**, *24*, 773.
(7) Findlay, S. P.; Small, L. F. *J. Am. Chem. Soc.* **1950**, *72*, 3249.

0022-2623/85/1828-0461$01.50/0