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Synthesis of Derivatives of NK109, 7-OH Benzo[*c*]phenanthridine Alkaloid, and Evaluation of their Cytotoxicities and Reduction-Resistant Properties

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Abstract—The N_5–C_6 double bond of NK109 (an antitumor benzo[*c*]phenanthridine alkaloid) is easily reduced under biological environment. To suppress the inactivation caused by reduction, we synthesized 5-, 6-, and 8-substituted NK109. 5-Substituted derivatives (**4a**–**c**) were reduced more easily than NK109. 6-Substituted ones (**10a**–**f**) inhibited biological reduction, but showed weak cytotoxic activity. 8-*O*-Substituted ones (**13a**–**h**), especially 8-*O*-hydroxyethyl NK109 (**13d**), suppressed biological reduction and exhibited strong cytotoxic activity.

Introduction

NK109 (**1**) is a synthetic benzo[*c*]phenanthridine alkaloid (BCPA) with significant antitumor activity. It exhibits stronger activity against P388 leukemia than the widely known BCPAs, nitidine and fagaronine.[1] NK109 inhibits topoisomerase II (Topo II) by stabilization of a cleavable complex of DNA and Topo II,[2] and is effective against several drug-resistant tumor cell lines.[3] Thus, we conducted a clinical trial of NK109 in Japan.[4] Although NK109 possesses sufficient antitumor activity for clinical trial, it is easily metabolized under biological environment. A metabolic investigation of NK109 in mice revealed an important metabolic pathway to be reduction to a 5,6-dihydro NK109 **2** (Fig. 1). NK109 strongly inhibits the growth of tumor cells in vitro. However, a high dose of NK109 is required to exhibit strong antitumor activity in vivo,[1] because the major metabolite (**2**) is inactive. In order to improve the metabolic stability of NK109, we decided to synthesize derivatives of NK109 with reduction-resistant property. In this paper, we report the synthesis of NK109 derivatives having 5-, 6-, and 8-*O*-substituents. We also report their cytotoxicities against HeLa S3 cells and reduction-resistant properties using human liver S9 mix.

Results and Discussion

The presence of a hydroxy group at the 7-position of NK109 is unique among BCPAs and is essential for exhibition of antitumor activity.[1,5] We thus modified 5-CH_3, 6-H, and 8-OCH_3 of NK109 with retaining the 7-OH group. First, benzo[*c*]phenanthridine **3**[6] was treated with several alkylating agents and followed by debenzylation with acid to yield *N*-substituted derivatives **4a**–**d** (Table 1).

We next synthesized 6-substituted NK109 (Scheme 1) from compound **5**, which is a synthetic equivalent of quaternary BCPA **6** soluble in organic solvent.[6] Compound **5** was treated with several Grignard reagents in THF to give 6-alkyl intermediates **7**. They are 5,6-dihydro BCPA and, therefore, aromatized with MnO_2 in toluene. When R^2 is an alkyl group, the oxidation proceeded with removal of the *N*-methyl group to yield benzo[*c*]phenanthridine **8**. After **8** was *N*-methylated again with methyl trifluoromethane sulfonate, the product was debenzylated with HCl to give 6-alkyl NK109 **10a**–**d** (Table 2). When R^2 is an aryl group, the oxidation proceeded without removal of the *N*-methyl group to yield **9**. It was debenzylated with HCl to give 6-aryl NK109 **10e**–**f**.

Finally, we synthesized 8-*O*-substituted NK109 (Scheme 2). A quaternary BCPA **11**, synthesized by the synthetic procedure of NK109,[6] was reduced to 7,8-dihydroxy BCPA **12** by catalytic hydrogenation. It was treated with several alkyl halides in the presence of potassium carbonate in acetone.[7] The reaction mixture included

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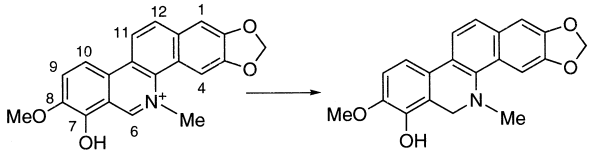
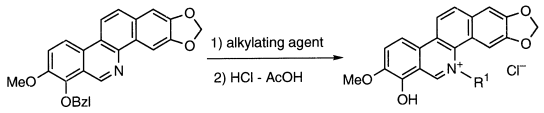


1 (NK109) 2

Figure 1. Reduction of NK109 (**1**).

Table 1. Synthesis of 5-substituted NK109 (**4**)



3 4

Alkylating agent[a]	Product (R^1)	Yield (%)
EtOTf	**4a** (Et)	75
ONBSP	**4b** (Pr)	14
c-PrBr-AgOTf	**4c** (Allyl[b])	17
$MeOCOCH_2OTf$	**4d** (CH_2CO_2H)	81

[a]Abbreviations of alkylating agents are as follows: EtOTf, ethyl trifluoromethanesulfonate; ONBSP, *n*-propyl 2-nitrobenzenesulfonate (see ref 6); *c*-PrBr, *c*-propyl bromide.
[b]The reaction of *c*-propyl cation and **3** yielded *N*-allyl derivative, not *N*-*c*-propyl derivative.

mono-*O*-alkylated and di-*O*-alkylated products which, after oxidation with DDQ, were isolated by silica gel chromatography to give **13**–**15** (Table 3). Compounds **13** and **14** are easily detected by TLC monitoring, because they show purple and yellow spots on the TLC plate, respectively.

We evaluated reduction-resistant properties of the NK109 derivatives **4**, **10** and **13**, using human liver S-9 mix. First, we detected their reduction product peaks by HPLC analysis.[8] NK109 was reduced quantitatively with $NaBH_3CN$ to 5,6-dihydro NK109,[1] and **4**, **10**, **13** were also converted with $NaBH_3CN$ to the corresponding dihydro derivatives almost quantitatively on HPLC chromatogram. Based on the retention time of each product, we identified the converted peak to be the corresponding reduced product. Subsequently, NK109 and its derivatives were treated with human liver S-9 mix and NADPH.[9] After incubation at 37 °C for 30 min, they were analysed on HPLC. Under this condition, NK109 was reduced partially and peaks of both intact

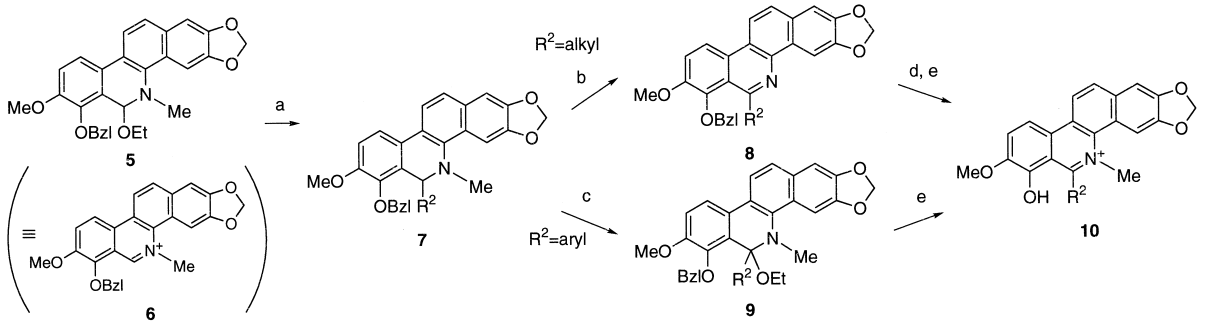
Table 2. Yield of 6-alkylation of NK109

Substituent (R)	Alkylating agent	Product (yield %) **8**, **9**	Product (yield %) **10**
Me	MeMgBr	**8a** (53)	**10a** (91)
Et	EtMgBr	**8b** (54)	**10b** (35)
Pr	PrMgBr	**8c** (59)	**10c** (28)
c-Pr	*c*-PrMgBr	**8d** (65)	**10d** (32)
Ph	PhMgBr	**9e** (87)	**10e** (75)
p-(OMe)-Ph	*p*-(OMe)-PhMgBr	**9f** (54)	**10f** (93)

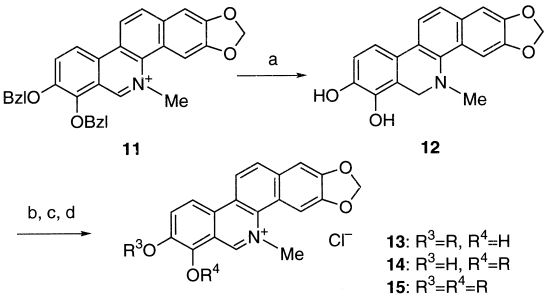
and the reduced product were observed on the HPLC chromatogram. The synthetic derivatives were also analyzed in the same way. After calculating the ratio of the reduced products, we judged the reduction-resistant property based on comparison of the reduction rate of NK109 and those of derivatives. These results are shown in Table 4. Although the reduction rate of 5-substituted derivatives **4** is higher than that of NK109, that of 8-*O*-substituted derivatives **13** is lower than that of NK109. In the case of 6-substituted derivatives **10**, no reduced products were observed. Thus, 8-*O*-substituents partially contribute to resistance to the biological reduction and 6-subsituents completely do. On the other hand, 5-substituents accelerated the reduction (Fig. 2).

We also evaluated the cytotoxicities of NK109 derivatives against HeLa S3 cells according to the previously reported procedures.[1] These results are also listed in Table 4.

We synthesized several derivatives of NK109 aiming at those with reduction-resistant property. 6-Substituted derivatives **10** completely became resistant to the biological reduction independent from the kind of substituent; however, they also lost their cytotoxic activity. It is generally believed that the immonium group of BCPA is necessary for their antitumor activity,[10] and thus the immonium group would be concerned in the mode of action.[11] In the case of **10**, 6-substituents will affect their antitumor activity. 8-*O*-Substituted derivatives **13** partially inhibit biological reduction. Derivatives with bulky hydrophobic substituents such as an isopropyl or a benzyl group (**13a** and **13h**) showed weak activities, probably because these substituents are not suitable for the environment of the binding site of Topo II. Those with hydrophilic substituents such as hydroxyethyl (**13c** and **13d**) showed similar activity as NK109. 5-Substituted



Scheme 1. Synthesis of 6-substituted NK109 (**10**). Reagent: (a) R^2MgBr, THF; (b) MnO_2, toluene; (c) DDQ, CH_2Cl_2; (d) MeOTf, toluene; (e) HCl, AcOH.



Scheme 2. Synthesis of 8-*O*-Substituted NK109 (**13**). Reagent: (a) H_2, Pd-C, MeOH–H_2O; (b) R-Br, K_2CO_3, acetone; (c) DDQ, CH_2Cl_2; (d) HCl.

Table 3. Yield of 7- and 8-alkylation of *O*-demethyl-NK109

Substituent (R)	Alkylating Agent	Product (yield%) C_8-OR **13**	C_7-OR **14**	$C_{7,8}$-$(OR)_2$ **15**
i-Pr	*i*-PrBr	**13a** (14)	**14a** (4)	—
Allyl	Allyl-Br	**13b** (6)	—	**15b** (52)
$(CH_2)_2OAc$	$Br(CH_2)_2OAc$	**13c** (9)	**14c** (3)	**15c** (2)
$(CH_2)_2OH$	—	**13d**[a] (5)	—	—
CH_2CONH_2	$ClCH_2CONH_2$	**13e** (4)	**14e** (2)	**15e** (6)
CH_2CO_2Me	$BrCH_2CO_2Me$	**13f** (4)	—	**15f** (13)
CH_2CO_2H	—	**13g**[b] (4)	—	**15g**[b] (1)
Bzl	—	**13h**[c]	—	—

[a]Obtained together with **13c**–**15c** by the reaction of **12** and $Br(CH_2)_2OAc$.
[b]Obtained together with **13f** and **15f** by the reaction of **12** and $BrCH_2CO_2Me$.
[c]Obtained by partial debenzylation of **11**: compound **11** and trifluoroacetic acid were warmed at 50 °C for 1 h to give **13h** (33%).

Table 4. Evaluation of substituted NK109

Compound	Reduction-resistant property Relative rate[a]	Judgement[b]	Cytotoxicity IC_{50} (μM)
NK109	1.0		0.32
4a	3.4	−	0.40
4b	1.6	−	0.76
4c	2.6	−	0.49
4d	0.21	+	>24
10a	n.d.[c]	++	2.9
10b	n.d.	++	11
10c	n.d.	++	2.9
10d	n.d.	++	6.5
10e	n.d.	++	17
10f	n.d.	++	16
13a	0.81	+	2.4
13b	2.4	−	0.71
13c	n.d.	+[d]	0.48
13d	0.08	+	0.52
13e	0.57	+	1.9
13f	0.96	+	15
13g	0.42	+	>24
13h	0.42	+	17

[a]Relative reduction rate to NK109: (reduction rate of derivative)/(reduction rate of NK109). The reduction rate is calculated as follows: R/(R + I); 'R' is the peak area of reduced product under the HPLC chromatograph, and "I" is the peak area of intact compound.
[b]Defined as follows: ++, reduced product peak is not detected (relative rate = 0); +, relative rate < 1; −, relative rate > 1.
[c]The reduced product peak was not detected.
[d]Although reduced product peak of **13c** was not detected, its metabolites, **13d** and reduced product peak of **13d**, were observed.

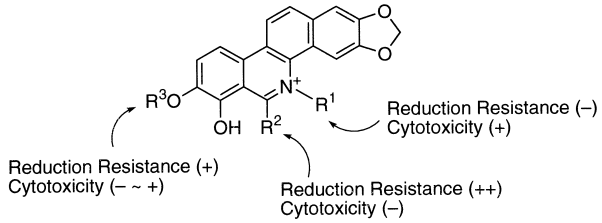


Figure 2. Substituent effect on activity.

derivatives showed strong activity. However, they were reduced faster than NK109. In view of the reduction rate and synthetic difficulties,[12] 5-substituted ones are not superior to NK109. We also found that derivatives with a carboxy group (**4d** and **13g**) lost activity. The reason would be the poor solubility in aqueous media and poor penetration into the cells.

In conclusion, derivatives with substituents at the 6-position completely became resistant to the biological reduction. However, they are unsatisfactory because they showed weak cell growth inhibitory effect. The substituent at the 8-position suppresses biological reduction. Unless the 8-*O*-substituent is not bulky and hydrophobic, they show similar activity as NK109. 8-*O*-Hydroxyethyl derivatives of NK109 (**13c** and **13d**) possess both reduction-resistant properties and strong cytotoxicities, and are therefore expected as new lead compounds to back-up NK109.

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7. In other solvents such as MeOH, CH_3CN, and THF, no alkylating product was obtained.
8. HPLC conditions: Column, Inertsil C8 6.0×200 mm; mobile phase, 1% H_3PO_4 + 5 mM *n*-Bu_4NBr:CH_3OH (65:35); flow rate, 1.5 mL/min; detection, UV (344 nm).
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11. For example, the interaction with tublin: Wolff, J.; Knipling, L. *Biochemistry* **1993**, *32*, 13334.
12. The *N*-alkylation of BCPA is affected by the size of substituent because of the steric effect of 4-H. Therefore, even the rate of *N*-ethylation is much slower than that of *N*-methylation.