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Graphical Abstract

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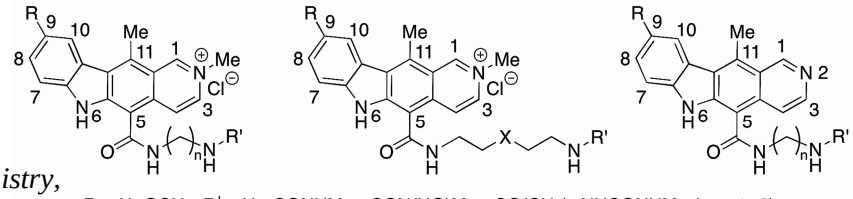
Department of Pure and Applied Chemistry, Faculty of Science & Technology, Tokyo University of Science (RIKADAI), Noda, Chiba 278-8510, Japan



R = H, OCH_3; R' = H, -CONHMe, -CON(NO)Me, $-CO(CH_2)_mNHCONHMe$ (m = 1, 2)
n = 2 - 5; X = O, NH

Synthesis, potent cytotoxic activity, and odd-even effect

Synthesis and cytotoxic activity of novel 11-methyl-6*H*-pyrido[4,3-*b*]carbazole derivatives linked to amine, *N*-methylurea, and *N*-methyl-*N*-nitrosourea moieties with various types of carbamoyl tethers at the C-5 atom

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Department of Pure and Applied Chemistry, Faculty of Science & Technology, Tokyo University of Science (RIKADAI), Noda, Chiba 278-8510, Japan
Fax: +81-4-7123-9326
E-mail: konaka@rs.noda.tus.ac.jp
Homepage: http://www.tus.ac.jp
Supporting information for this article is available on the WWW under http://.

Keywords: Ellipticine; 6*H*-Pyrido[4,3-*b*]carbazole; Synthesis; Cytotoxic activity; Cell selectivity; Odd-even effect; Sarcoma-180; HeLa S-3; NIH3T3; L1210

Abstract—Thirty-five types of novel pyridocarbazoles (5-(*N*-alkyl)carbamoyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazoles and 5-(*N*-alkyl)carbamoyl-2,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride derivatives), that were conjugated with amine, *N*-methylurea, and *N*-methyl-*N*-nitrosourea moieties through alkyl-, oxyalkyl-, and iminoalkylcarbamoyl linkers, were synthesized by a series of reactions of methyl 11-methyl-6*H*-pyrido[4,3-*b*]carbazole-5-carboxylates with polymethylenediamine (*n* = 2-5), *p*-nitrophenyl *N*-methylcarbamate, and *N*-methyl-*N*-nitrosocarbamate in high yields, and their cytotoxic activities were evaluated against Sarcoma-180, NIH3T3, HeLa S-3, and L1210 cell lines. These compounds exhibited potent cytotoxic activity (IC_{50} = 1.6 – 50 μM) and odd-even alternation effect. 9-Methoxy-2,11-dimethyl-5-((2-(3-methyl-3-nitrosoureido)ethyl)carbamoyl)-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride exhibited the most potent cytotoxic activity (IC_{50} = 0.15 μM) and cell selectivity against HeLa S-3. Some of the un-charged 5-(*N*-alkyl)carbamoyl-6*H*-pyrido[4,3-*b*]carbazole derivatives, which were conjugated with the amine and *N*-methyl-*N*-nitrosourea moieties through a dimethylene spacer, also exhibited potent cytotoxic activity (IC_{50} = 0.43 – 2.4 μM) and remarkable cell selectivity as well as ellipticine, 9-hydroxyellipticine, and the starting methyl ester of the pyridocarbazole-5-carboxylate (IC_{50} = 0.30 – 2.2 μM).

1. Introduction

A great deal of interest has been generated by indole alkaloids (ellipticines[1] and β-carbolines[2]) because of their intercalation,[1h] antitumor properties,[1c,d,f] anti-HIV activities, and inhibition of RNA polymerase I transcription.[1g] In 1959, ellipticine (**1**) was first identified as a compound in the leaves of a small tropical evergreen, *Ochrosia elliptica* Labill[3] (Fig. 1). Ellipticine and its derivatives cause the selective inhibition of p53 protein phosphorylation in several human cancer cell lines,[4a] induced apoptosis of cancer cell lines,[4b] and this is correlated with their cytotoxic activity. These compounds also uncouple mitochondrial oxidative phosphorylation,[5] thereby disrupting the energy balance of cells. Further, these compounds are known to react with DNA via an intercalation process,[1h] which may account for their cytotoxicity,[6] and also inhibits DNA topoisomerase-II activity.[1a] We used deflection spectroscopy to determine the interaction mode of ellipticine and its derivatives with DNA.[7] Ellipticine has shown intercalation and a cytotoxic activity that resembles that of β-carbolines.[8] The unique structural features, limited toxic side effects, and complete lack of hematological toxicity of these compounds have prompted chemists to study the synthesis of a number of their analogues in pharmacological evaluations.[9]

The total synthesis of ellipticine (**1**) was first achieved in 1959.[10a] Since then, many groups have synthesized ellipticine and its derivatives.[10,11] Previously, we developed a novel and efficient method for the synthesis of **1** via Suzuki-Miyaura coupling.[12] Several simple structural modifications to ellipticine derivatives have resulted in compounds that have shown increases in cytotoxic activity.[13] A low level of water solubility at physiological pH, as well as systemic toxicity, has prevented the use of **1** as a therapeutic agent. The introduction of a positive charge in **1** improved water solubility and made a profound difference in its biological activity.[14] For example, the quaternization of the endocyclic nitrogen at the N5 of 5-aza-ellipticine[15] and the nitrogen at the N2 of ellipticine[16] with a methyl group was found to interact with DNA through intercalation, which resulted in a high affinity for binding with DNA.

Recently, we reported the syntheses of the *N*-methyl-*N*-nitrosourea conjugates of ellipticin-2-ium chloride, 9-hydroxyellipticin-2-ium chloride, and 5-methoxycarbonyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride derivatives, linked by an oxydiethylene unit and a pentamethylene unit at the 2 position of the nucleus.[11,17] These compounds have exhibited potent cytotoxic activity against the human cervical cancer cell line HeLa S-3 and have shown solubility in water that was higher than that of ellipticine itself, which is based on results reported on celliptium and datlliptium.[18]

The potential for these compounds[11, 17] encouraged us to focus on the development of novel ellipticine analogues (**2**) that might exert stronger cytotoxic activity via the C5 introduction of a carbamoyl group possessing various types of linkers with an *N*-methyl-*N*-nitrosourea moiety (Fig. 1).

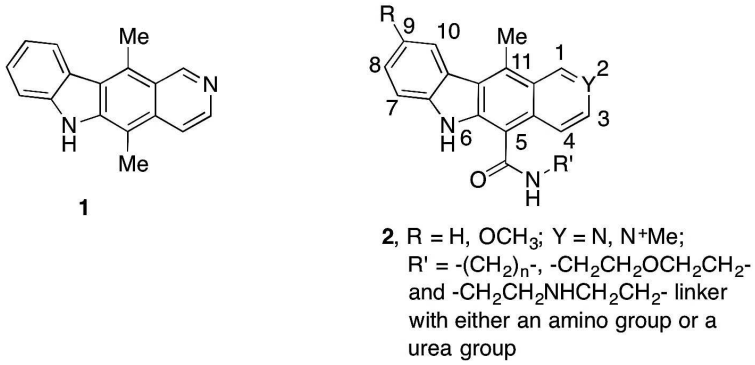


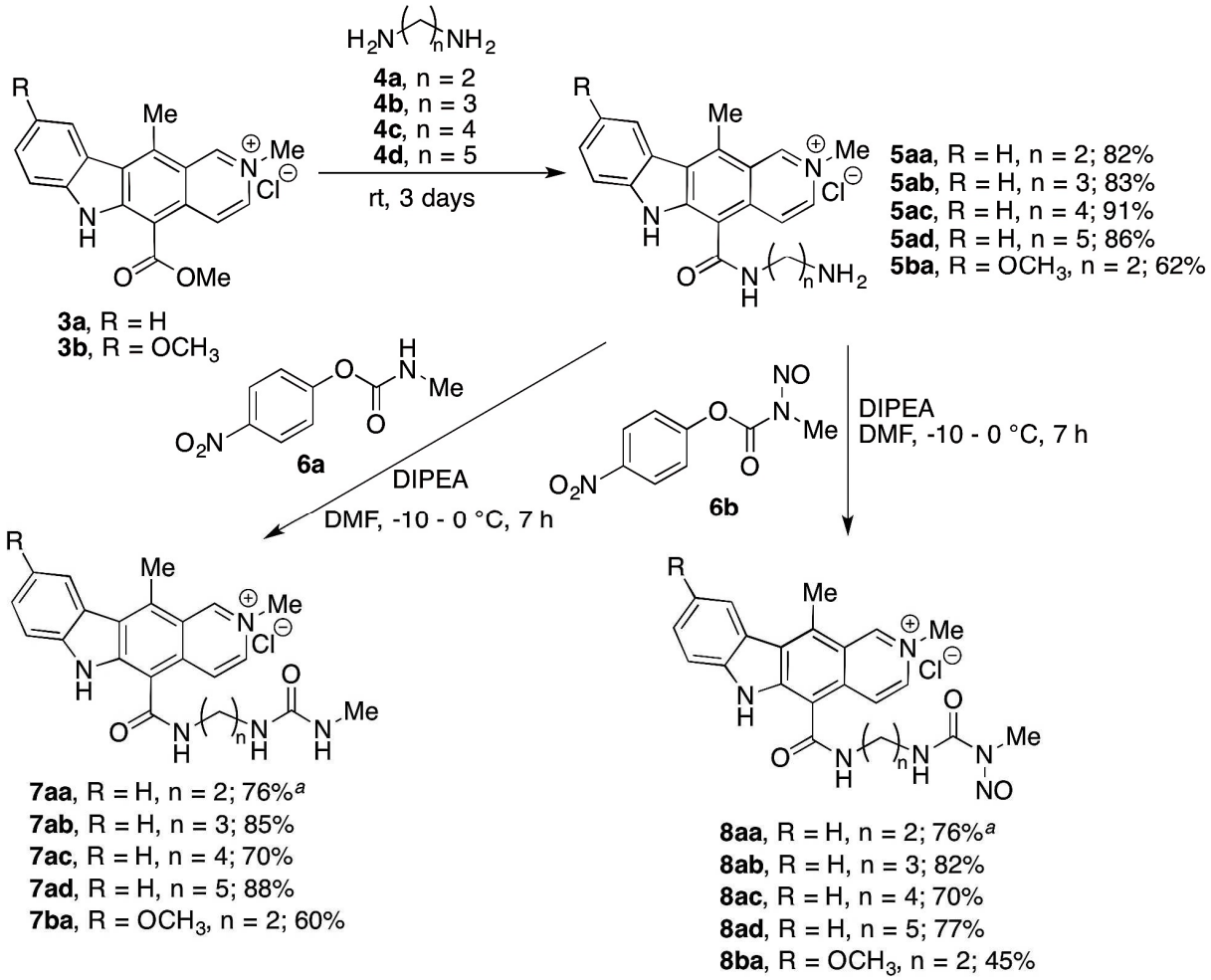
Fig. 1. Chemical structures of Ellipticine (**1**) and 5-alkylcarbamyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazole derivatives **2**, which were synthesized in this work.

2. Results and discussion

2.1. Chemistry

*2.1.1. Synthesis of 5-(N-alkyl)carbamoyl-2,11-dimethylpyridocarbazol-2-ium chloride derivatives linked to amine, urea, and nitrosourea moieties **5**, **7**, **8**, **10**, **11**, and **12***

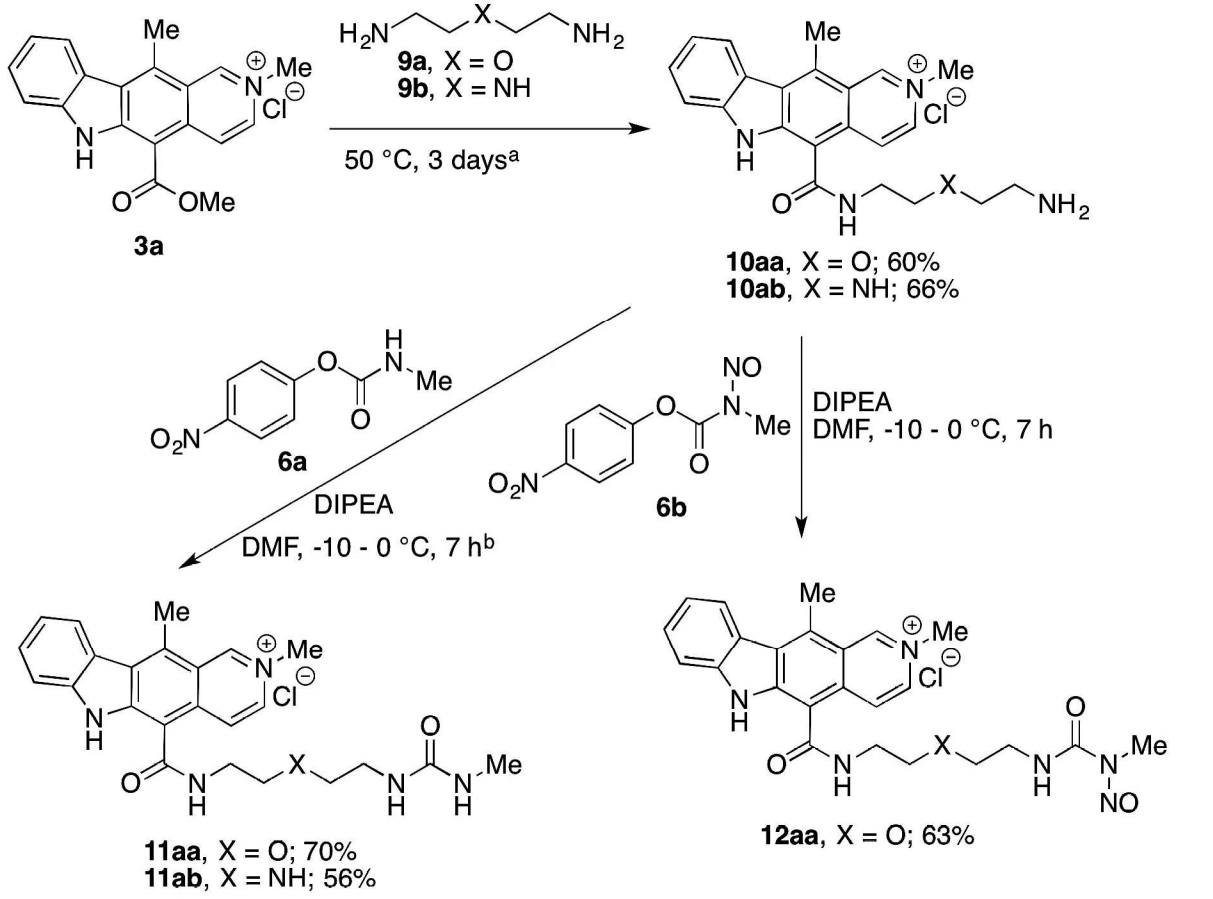
We began with the preparation of 5-(2-aminoethyl)carbamyl-2,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride **5aa**. The starting methyl ester **3a**, which was synthesized using a previously reported method,[17] was allowed to react with ethylenediamine (**4a**) at room temperature for 3 days to produce the *N*-(2-aminoethyl)amide **5aa** in an 82% yield (Scheme 1). Under similar conditions, the corresponding *N*-(ω-aminoalkyl)amides **5ab**, **5ac** and **5ad** were obtained in 83, 91 and 86% yields, respectively. The 9-methoxy derivative **5ba** was also synthesized from the corresponding 9-methoxy derivative **3b** and **4a** in a 62% yield. When coupled (in DMF at -10 to 0 ^{o}C for 7 h) with the *p*-nitrophenyl (or 2,5-dioxopyrrolidin-1-yl) *N*-methyl- and *N*-methyl-*N*-nitrosocarbamates **6a** and **6b**,[19] the *N*-(ω-aminoalkyl)amides **5** afforded the desired urea compounds **7aa**-**ba** and **8aa**-**ba** in 60-88 and 45-82% yields, respectively[20] (Scheme 1). TLC and NMR analysis of the reaction mixtures for **7aa**-**ba** and **8aa**-**ba** indicated no formation of by-products with the noted exception of the unreacted starting materials and the final product, which indicated that selective coupling was possible between the primary amines with *p*-nitrophenyl *N*-methylcarbamates **6a**,**b** only under these developed conditions.



[a] The corresponding 2,5-dioxopyrrolidin-1-yl *N*-methylcarbamates were used in stead of **6a,b**.

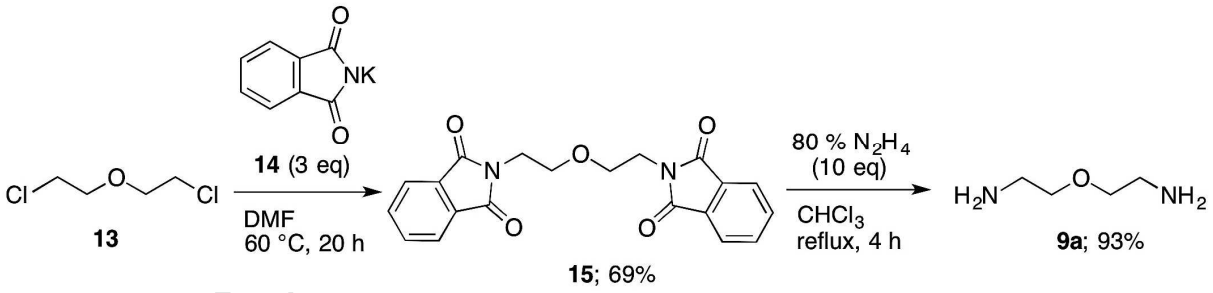
Scheme 1. Synthesis of 5-carbamylpyridocarbazol-2-ium urea derivatives with the polymethylene-linkers, **7** and **8**

In previous work, an ethoxyethyl linker (ether-linker) was more effective in cytotoxic expression than the corresponding pentamethylene linker.[17] Therefore, we planned to synthesize the corresponding 5-(*N*-(2-ethoxy)ethyl)carbamoyl derivatives **11aa** and **12aa**, and 5-(*N*-(2-(ethylamino)ethyl))carbamoyl derivative **11ab** (Scheme 2). The reaction of **3a** with an ether-linker **9a** and an imino-linker **9b** was performed at 50 °C for 3 days to give the corresponding amide compounds in 60 and 66% yields, respectively. The ether-linker **9a** was synthesized from bis(2-chloroethyl) ether (**13**) and potassium phthalimide (**14**) in good to high yields using the previously reported similar method[17] (Scheme 3).



[a] The reaction of **9b** was performed under irradiation of microwave for 2 h.
[b] The reaction of **10ab** was performed at -60 °C for 11 h.

Scheme 2. Synthesis of 5-carbamylpyridocarbazol-2-ium urea derivatives with ether- and imino-linkers, **11** and **12**

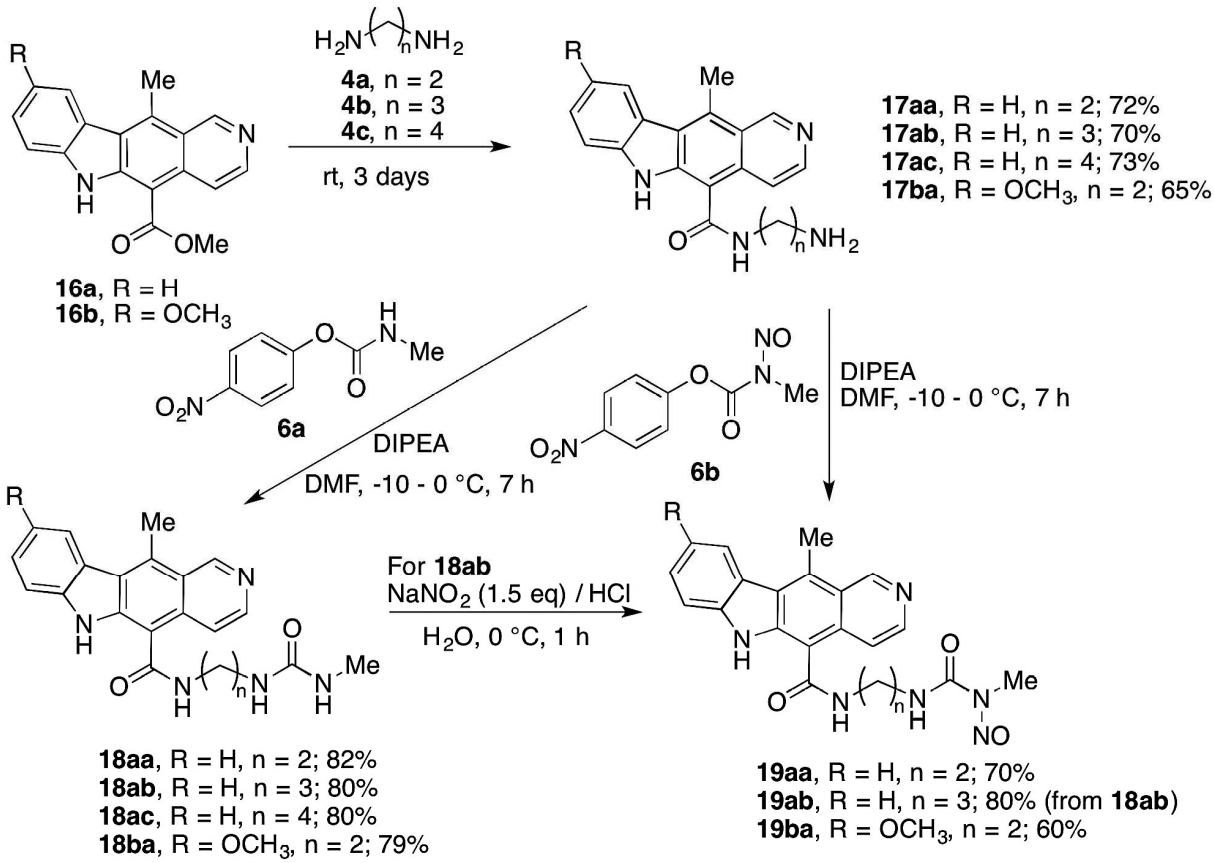


Scheme 3. Synthesis of 2,2'-oxybis(ethylamine) (**9a**)

*2.1.2. Synthesis of 5-carbamoylpyridocarbazole N-nitrosourea derivatives **18** and **19***

Quarternized pyridocarbazol-2-ium salts are more soluble in water than the un-quarternized counterparts. However, the cellular uptake of these positively charged compounds was affected by the membrane potential,[16c,21] and the positive charge was expected to influence their membrane permeability. In order to confirm the effect, un-quarternized 5-carbamoylpyridocarbazoles were synthesized via a method similar to the one shown in Scheme 1. The actual method is shown in

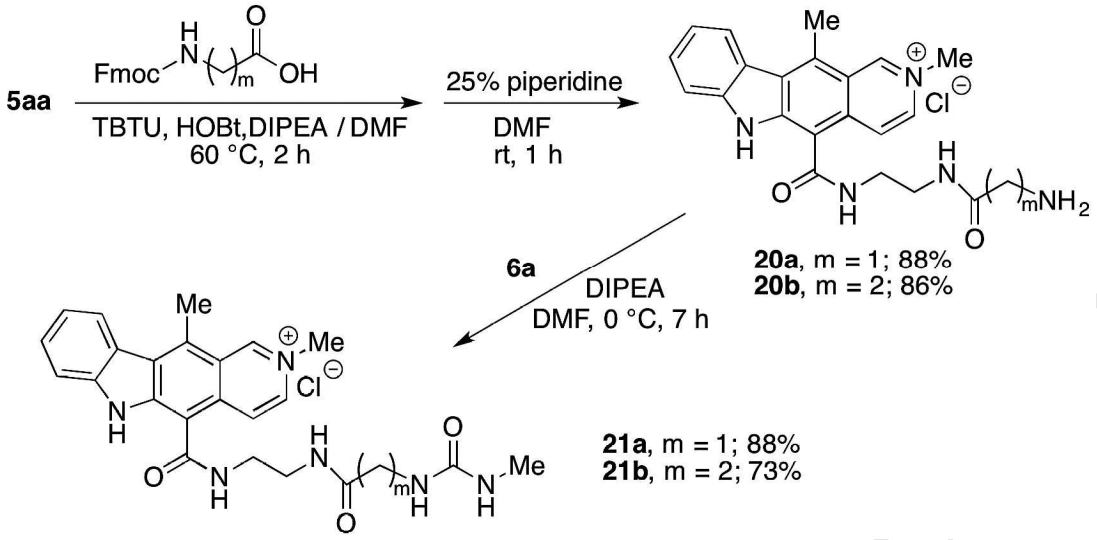
Scheme 4. A reaction series of methyl pyridocarbazole-5-carboxylates **16a,b** with diamines **4a-c** gave the corresponding amides **17aa**, **17ab**, **17ac**, and **17ba** (72, 70, 73, and 65%, respectively), urea derivatives **18aa**, **18ab**, **18ac**, and **18ba** (82, 80, 80, and 79%, respectively), and *N*-nitrosourea derivatives **19aa** and **19ba** (70 and 60%, respectively). The compound **19ab** was synthesized from **18ab** by an alternative method, nitrosation with nitrous acid at 0 °C in an 80% yield.



Scheme 4. Synthesis of 5-carbamoylpyridocarbazole-urea derivatives **18** and **19**

*2.1.3. Synthesis of amine- and urea-conjugates of pyridocarbazol-2-ium derivatives with peptide linkers **20a**,**b** and **21a**,**b***

In order to increase the hydrogen-bonding ability, a peptide group was added on the terminal amino group of **5aa**. The introduction of the peptide unit was accomplished via the coupling of **5aa** with Fmoc-amino acid in high yields. For example, the compound **5aa** was treated with Fmoc-glycine in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIPEA) in DMF leading to the formation of the peptide **20a** in an 88% yield after the deprotection of the Fmoc group by the action of piperidine (Scheme 5). With the analogues, the reaction with Fmoc-β-alanine proceeded smoothly to give the corresponding peptide **20b** in an 86% yield. Each terminal amino group of the peptides **20a**,**b** was transformed to the ureido group by the action of *p*-nitrophenyl *N*-methylcarbamate **6a** to give the corresponding urea derivatives **21a**,**b** in high to good yields (88 and 73%, respectively).



Scheme 5. Synthesis of amine and urea conjugates of pyridocarbazol-2-ium chloride derivatives with peptide-linkers **20a**,**b** and **21a**,**b**

2.1.4. Characterization of the product structures

The structures of all the synthesized compounds in schemes 1 - 5 were confirmed based on their ^{1}H and ^{13}C NMR and mass spectroscopic data. The aromatic protons of the products **5**, **7**, **8**, **10**, **11**, **12**, **17**, **18**, **19**, **20**, and **21** were showing the ^{1}H NMR signals in the range of δ 11.25-6.06[12,10g,22] and methyl protons of $N^{+}CH_3$ in compounds **5**, **7**, **8**, **10**, **11**, **12**, **20**, and **21** appeared as singlets in the range of δ 4.50-3.90,[23] which confirmed the basic pyridocarbazol-2-ium skeleton in these molecules. The methylene protons of the methylene- and ether-linkers in compounds **5ba**, **10aa**, **10ab**, **17aa**, and **17ba** resonated at δ 3.91-3.07 indicating a coupling with a linker skeleton.[24] The appearance of methyl protons for $NHCH_3$ at δ 2.63-1.66 in compounds **7**, **11**, **18**, and **21**, and of methyl protons for $N(NO)CH_3$ at δ 3.33-2.00 in compounds **8**, **12aa**, and **19** confirmed the *N*-methylurea moiety in these compounds.[25] Further, ^{13}C NMR results supported the proposed structures for these synthesized compounds (see Experimental). In addition, the presence of $[M^{+} - Cl^{-}]$ for compounds **5**, **7**, **8**, **10**, **11**, **12aa**, **20**, and **21**, and [M + H] for compounds **17**, **18,** and **19** at the respective *m/z* values, further confirmed their proposed structures.

2.2. Evaluation of cytotoxic activity

2.2.1. Cytotoxic activity of pyridocarbazol-2-ium chloride and pyridocarbazole derivatives against Sarcoma-180, NIH3T3, and HeLa S-3 cell lines; substituent effects

Most of the newly synthesized pyridocarbazole derivatives were screened via bioassay. The cytotoxic activity was evaluated using an MTT assay against Sarcoma-180 cancer cells and NIH3T3 normal cells[26] originating in mouse cell lines and a human cervical cancer cell line HeLa S-3. The results were summarized in Table 1 together with the cytotoxic activity of *cis*-diamminedichloro-

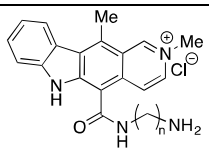
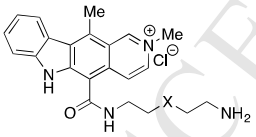
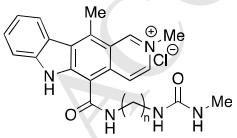
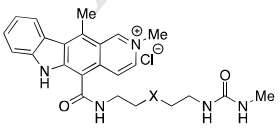
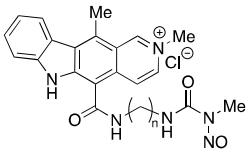
platinum(II) (CDDP) as a positive control. Firstly, we applied a series of 5-(ω-aminoalkyl)carbamoyl-2,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride derivatives **5aa**-**ad**, **10aa**, and **10ab**, in which the length of the methylene tether and the central element in the tether were changed (entries 1-6). The pyridocarbazolium derivatives **5ab** and **5ad** had an odd number of methylene units and showed higher activity against all cell lines than the derivatives **5aa** and **5ac**, which had an even number of methylene units (entries 1-4 except for **5ad** and **5ac** against HeLa S-3). In order to make the relationship understandable, IC_{50} values of **5aa**-**ad** were plotted against the number of the methylene units *n* in Fig. 2 (left-side panel; compounds **5**). As can be seen in Fig. 2, the compounds **5** exhibited odd-even alternation in the cytotoxic activity with respect to the methylene chain length. A similar odd-even effect was also observed in the cytotoxic activity of the corresponding *N*-methylurea and *N*-methyl-*N*-nitrosourea conjugates, **7aa**-**ad** (Fig. 2; right-side panel; compounds **7**) and **8aa**-**ad**, against mouse cell lines (entries 7-10 and 14-16). The lone exception was the activity of **8aa** (entry 13). In the activity against HeLa S-3, however, similar effect was not observed. Such odd-even effect has been reported in some biological activities; for example, a cytokinin activity of N^6-(ω-phenylalkyl)adenines and the related compounds,[27a] an cytoprotective activity of *N*-alkylisatins on the apoptosis of PC12 cells induced by H_2O_2,[27b] and a sex attractant activity of the cuticular hydrocarbons for Horn fly.[27c] Whereas the odd-even alternation in the cytokinin activity of ω-phenylalkyl derivatives of N^6-substituted adenines was reportedly not related to their lipophilicity but is related to a specific role of the ω-phenyl group for cytokinin-receptor binding,[27a] the alternate cytoprotective-activity of *N*-alkyl substituted isatin derivatives on the apoptosis of PC12 cells was not clarified.[27b] When CLogP values of **5aa**-**ad**, **7aa**-**ad**, and **8aa**-**ad** were plotted against the number of methylene units *n* in the linker, an odd-even alternation effect was observed as shown in Fig. 3. Therefore, as one of the plausible explanations, the odd-even effect in the cytotoxic activity may be related to an alternate change in the drug-lipophilicity, which influences the membrane permeability and the cellular uptake of the drug.[28] The odd-even effect in the cytotoxic activity is an interesting and noteworthy phenomenon in a structure-activity relationship, and is currently under investigation.

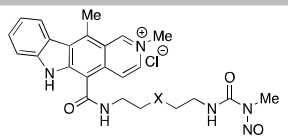
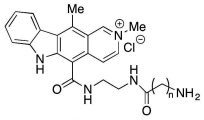
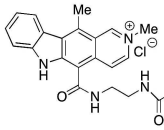
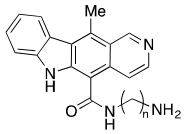
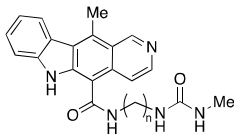
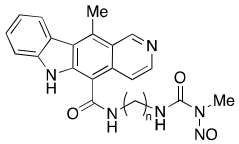
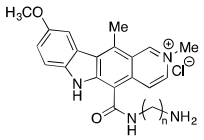
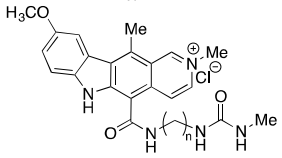
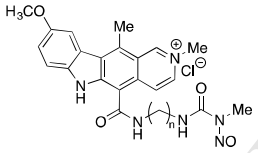
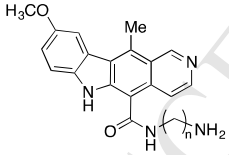
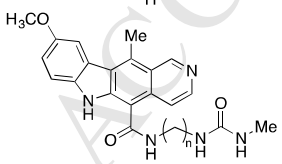
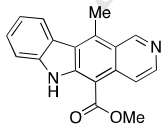
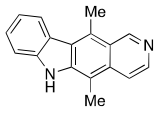
Next, replacement of the central methylene group of **5ad** (IC_{50} = 21 and 32 μM against Sarcoma-180 and NIH3T3, respectively) to an ether or imino group (X = O or NH in Scheme 2) suppressed its cytotoxic activity (**10aa**, IC_{50} = 48.0 and 60 μM; **10ab**, IC_{50} >100 and = 86 μM against Sarcoma-180 and NIH3T3, respectively; entries 4-6). On the other hand, the introduction of the peptide unit in the linker exhibited no effect on the cytotoxic activity against both mouse cell lines except for **20b** (entries 18-20). Unfortunately, the compound **20b** slightly decreased the cytotoxic activity against Sarcoma-180 cancer cells (0.6-fold *vs.* **5aa**) and slightly increased that against NIH3T3 normal cells (2.1-fold *vs.* **5aa**).

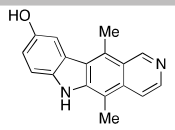
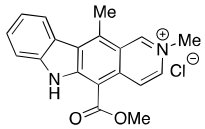
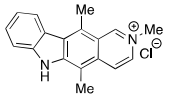
The ellipticin-2-ium derivatives reportedly showed selectivity for cancer cells and for the central nervous system, which had a mutated p53-type protein,[29] and effectively intercalated to DNA with large binding constants.[1h,7] The positive charge of the ellipticin-2-ium salts, however, had a

disadvantage in the cellular uptake of the drug as mentioned above. Therefore, we compared the cytotoxic activity of the quarternized 5-carbamoyl-2,11-dimethylpyridocarbazol-2-ium salts **5aa**, **7aa**, and **8aa** with the corresponding un-quarternized 5-carbamoyl-11-methylpyridocarbazole derivatives **17aa**, **18aa**, and **19aa** in order to confirm the decreasing cellular uptake of the charged drugs. The cytotoxic activities of the un-quarternized pyridocarbazole amine-conjugate **17aa** against all the tested cell lines were more potent than those of the corresponding quarternized pyridocarbazol-2-ium **5aa** by the power of 25-, 50-, and >42-fold against Sarcoma-180, NIH3T3, and HeLa S-3, respectively (entries 1 and 21), and were comparable to those of CDDP (entry 35). The 9-methoxypyridocarbazole amine-conjugate **17ba** extremely enhanced the cytotoxic activity against HeLa S-3 compared with the corresponding charged derivative **5ba** (96-fold; entries 24 and 27). Furthermore, although the power of increment was moderate, the un-quarternized *N*-methylurea- and *N*-methyl-*N*-nitrosourea-conjugates **18aa**, **19aa**, and **18ba** also increased the cytotoxic activities by >7-, >9-, and >7-fold for Sarcoma S-180, NIH3T3, and HeLa S-3 (entries 7 and 22); 15-fold for NIH3T3 (entries 13 and 23); 4-fold for HeLa S-3 (entries 25 and 28), respectively. In fact, brilliance level of the **17aa**-treated Sarcoma-180 cells (IC_{50} = 2.2 μM) was stronger than that of the **5aa**-treated Sarcoma-180 cells (IC_{50} = 55 μM) in fluorescence micrographs as shown in Fig. 4. These results may have been caused by the decreased cellular uptake of the positively charged drugs.[16]

Table 1. Cytotoxic activity of pyridocarbazol-2-ium and pyridocarbazole derivatives **1**, **3a**, **5**, **7**, **8**, **10**-**12**, and **16**-**23** against Sarcoma-180, NIH3T3, and HeLa S-3 cell lines

Entry	Compound	n / X	Compound No.	IC_{50} (μM)[a]		
				Sarcoma-180	NIH3T3	HeLa S-3
1		2	**5aa**	55 ± 7	70 ± 20	>100
2		3	**5ab**	26.0 ± 0.8	42 ± 2	>26
3		4	**5ac**	> 100	> 100	>100
4		5	**5ad**	21 ± 7	32 ± 9	>100
5		O	**10aa**	48.0 ± 0.8	60 ± 10	>100
6		NH	**10ab**	> 100	86.0 ± 8	Not tested
7		2	**7aa**	> 100	> 100	>100
8		3	**7ab**	34.0 ± 0.6	31 ± 2	>22
9		4	**7ac**	> 88	> 100	96 ± 2
10		5	**7ad**	80 ± 10	37 ± 4	>100
11		O	**11aa**	41 ± 4	18 ± 5	Not tested
12		NH	**11ab**	78 ± 2	45 ± 6	>100
13		2	**8aa**	3.0 ± 0.7	6.5 ± 0.8	6 ± 2
14		3	**8ab**	40 ± 2	Not tested	6 ± 2
15		4	**8ac**	> 82	66 ± 8	Not tested
16		5	**8ad**	37 ± 4	50 ± 10	Not tested

17		O	**12aa**	80 ± 10	39 ± 5	Not tested
18		2	**20b**	100 ± 20	34 ± 5	Not tested
19		1	**21a**	69 ± 8	70 ± 5	>100
20		2	**21b**	>100	68 ± 5	>100
21		2	**17aa**	2.2 ± 0.1	1.40 ± 0.05	2.4 ± 0.4
22		2	**18aa**	15 ± 3	11.0 ± 0.6	15 ± 1
23		2	**19aa**	1.6 ± 0.4	0.43 ± 0.09	Not tested
24		2	**5ba**	58 ± 8	Not tested	>100
25		2	**7ba**	Not tested	Not tested	32 ± 4
26		2	**8ba**	6 ± 2	Not tested	0.15 ± 0.03
27		2	**17ba**	Not tested	Not tested	1.04 ± 0.06
28		2	**18ba**	Not tested	Not tested	7.6 ± 0.7
29		none	**16a**	Not tested	0.5 ± 0.1	2.2 ± 0.1
30		none	**1**	2.0 ± 0.7	0.30 ± 0.08	2.1 ± 0.5[b]

31		none	**23**	0.44 ± 0.07	0.9 ± 0.2	Not tested
32		none	**3a**	1.0 ± 0.3	1.2 ± 0.4	1.2 ± 0.4
33		none	**22**	0.7 ± 0.2	2.0 ± 0.5	2.90 ± 0.02[b]
34	*N*-methyl-*N*-nitrosourea	none	MNU	130 ± 20	480 ± 20	>100
35	*cis*-[$PtCl_2(NH_3)_2$]	none	CDDP	1.0 ± 0.3	2.0 ± 0.3	2.1 ± 0.5

[a] MTT assay was used and IC_{50} values are reported as the means of three experiments; the standard deviations are given together. [b] Quoted from ref. 17

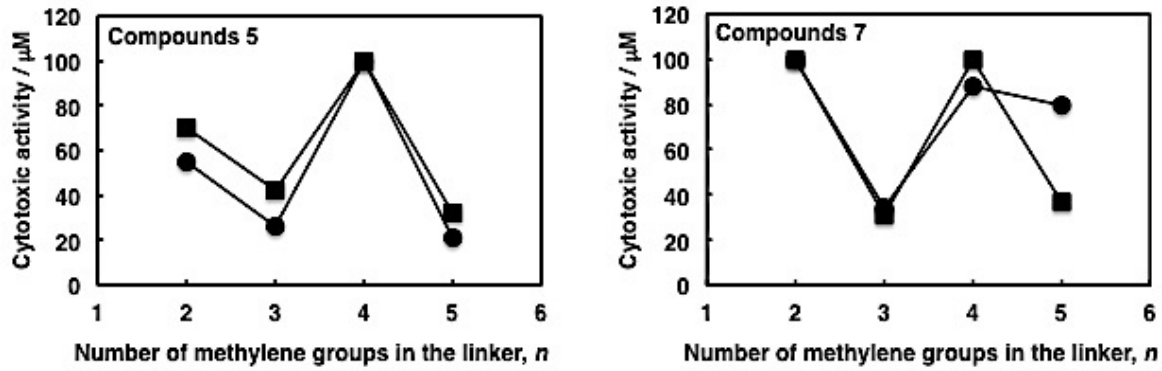


Fig. 2. Odd-even alternation in IC_{50} values of **5aa-ad** (the left-side panel) and **7aa-ad** (the right-side panel) against Sarcoma-180 (-●-) and NIH3T3 (-■-). IC_{50} value of >100 μM was temporarily replaced with 100 μM in the Figures.

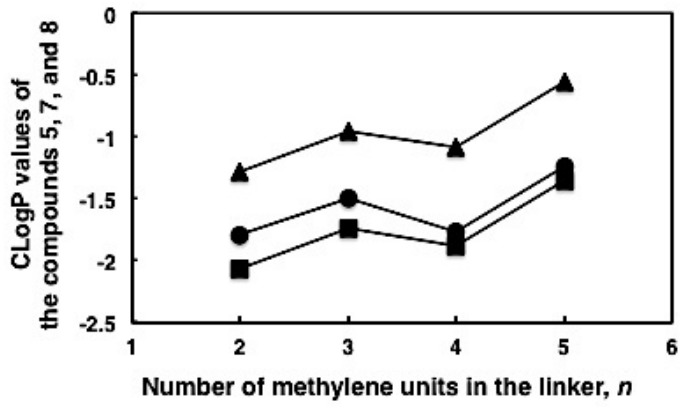


Fig. 3. CLogP values vs. number of methylene groups, *n*, in the linker of **5aa-ad** (-■-), **7aa-ad** (-●-), and **8aa-ad** (-▲-); **aa**: *n* = 2, **ab**: *n* = 3, **ac**: *n* = 4, **ad**: *n* = 5.

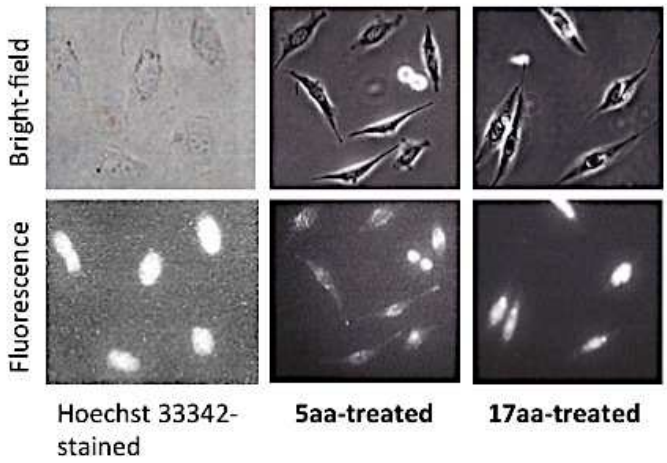


Fig. 4. Fluorescent micrographs of Sarcoma-180 mouse cancer cells, which were treated with 2,11-dimethylpyridocarbazol-2-ium amine-conjugate **5aa** (center) and 11-methylpyridocarbazole amine-conjugate **17aa** (right side) for 48 h. The left side panel was that of a control cells stained with Hoechst 33342.

Under physiological conditions, the 9-methoxyellipticines were reportedly *O*-demethylated leading to the formation of 9-hydroxyellipticines,[30] which exhibited higher cytotoxic activity (entry 31).[17] The drastically-increased cytotoxic activity by the introduction of the methoxy group was observed in the cytotoxic activities of **8ba** against HeLa S-3 (40-fold; Table 1, entries13 and 26). However, in the case of Sarcoma-180, there was no effect on the cytotoxic activity of **8ba** (entries 13 and 26) as well as that of **5ba** (entries 1 and 24).

Generally, introduction of an *N*-methyl-*N*-nitrosourea moiety to a drug induces its cytotoxicity.[20] The cytotoxic activities of the *N*-methyl-*N*-nitrosourea derivative **8ba** (IC_{50} = 0.15 μM against HeLa S-3, entry 26) and **19aa** (IC_{50} = 0.43 μM against NIH3T3, entry 23) were remarkably higher than those of the other tested compounds. This acceleration may have been caused by the synergy effect between the pyridocarbazole nucleus and the *N*-methyl-*N*-nitrosourea moiety. The cytotoxic activity of *N*-methyl-*N*-nitrosourea (MNU) itself against mouse cancer and normal cell lines was very low as shown in entry 34 as a negative control.

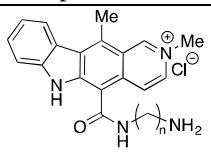
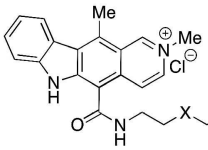
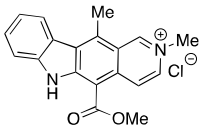
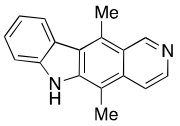
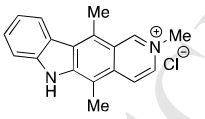
Replacement of the 5-methyl group of ellipticine (**1**) or 2-methylellipticin-2-ium chloride (**22**) by a methoxycarbonyl group did not affect the cytotoxic activity against all the tested cell lines (Table 1, entry 29 for **16a**; 32 for **3a**). In most case, transformation of the methyl ester group to the amide group decreased its cytotoxic activity.

2.2.2. *Cytotoxic activity of pyridocarbazol-2-ium and pyridocarbazole derivatives against L1210 mouse leukemia cells*

Some of the synthesized compounds were applied for the MTT assay against L1210 mouse leukemia cells. IC_{50} values for **1**, **3a**, **5ab**, **10aa**, **10ab**, and **22** were summarized in Table 2 together

with that of CDDP. The pyridocarbazolium chloride amine-conjugates **5ab**, **10aa** and **10ab** exhibited potent cytotoxic activities against L1210. Whereas the cytotoxic activities against L1210 of **10aa** and **10ab** (IC_{50} >50 and = 44 μM, respectively; entries 2 and 3 in Table 2) were not always lower than those against Sarcoma-180, NIH3T3, and HeLa S-3 cell lines, that of **5ab** was equal to or lower than those against Sarcoma-180, NIH3T3, and HeLa S-3 cell lines. Furthermore, the cell growth of L1210 was extremely suppressed by treatment with the methyl ester **3a**, ellipticine (**1**), and 2-methylellipticin-2-ium chloride (**22**) as well as those of Sarcoma-180, NIH3T3, and HeLa S-3 cell lines. The IC_{50} values of **3a**, **1**, and **22** were 0.6, 0.2, and 0.21 μM, respectively (entries 4-6), which were comparable to that of CDDP (0.37 μM, entry 7).

Table 2. Cytotoxic activity of pyridocalbazol-2-ium and pyridocalbazole derivatives **5ab**, **10aa**, **10ab**, **3a**, **1**, and **22** against L1210 mouse leukemia cells

Entry	Compound	n / X	No.	IC_{50} (μM)[a]
1		3	**5ab**	46 ± 3
2		O	**10aa**	>50
3		NH	**10ab**	44 ± 3
4		none	**3a**	0.6 ± 0.4
5		none	**1**	0.2 ± 0.3
6		none	**22**	0.21 ± 0.05
7	*cis*-$[PtCl_2(NH_3)_2]$	none	CDDP	0.37 ± 0.05

[a] MTT assay was used and IC_{50} values are reported as the means of three experiments; the standard deviations are given together.

2.3. Comparative study on the cytotoxic activity of pyridocarbazol-2-ium chlorides

2.3.1. Cell selectivity in the cytotoxic activity of 5-(N-alkyl)carbamoyl-2,5-dimethyl-6H-pyrido[4,3-b]carbazol-2-ium derivatives

Whereas the N2-methylated quarternary salts, **3a**, **8aa**, **8ba**, and **22**, were easily soluble in water

and exhibited extremely potent cytotoxic activity, the cellular uptake of these positively charged compounds was affected by the membrane potential,[16c,21] and the positive charge was expected to influence their membrane permeability. In fact, the uncharged pyridocarbazole **17aa** was dominant in the cellular uptake over the corresponding charged compound **5aa** as demonstrated by the fluorescence microscopic observation (Fig. 4). Although the compound **8ba** had a positive charge, it exhibited characteristic cell-selectivity against HeLa S-3 by the power of 40-fold compared with Sarcoma-180 (Table 1, entry 26). On the other hand, the uncharged compound ellipticine (**1**) exhibited the medium-intensity cell-selectivity against L1210 (10-fold vs. Sarcoma-180; see Table 2, entry 5 and Table 1, entry 30) and NIH3T3 (7-fold vs. HeLa S-3; Table 1, entry 30). Furthermore, both the uncharged compounds **19aa** and **16a** also exhibited the moderate cell-selectivity against NIH3T3 (4-fold vs. each of Sarcoma-180 and HeLa S-3 cell lines; Table 1, entries 23 and 29).

When the IC_{50} values of 5-(*N*-alkyl)carbamoyl-2,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium derivatives **5**, **7**, **8**, **10**, **11**, and **12** against Sarcoma-180 cancer cells were plotted against those of NIH3T3 normal cells, a significant linear relationship was observed as shown in Fig. 5. The regression line was expressed by an equation $y = 1.24x + 0.42$ (relation coefficient $r = 0.967$) with the exceptions of **7ad**, **11aa**, **11ab**, and **12aa**. The slope of the regression line was nearly equal to one. It means that most of the tested compounds did not exhibit cell selectivity. The compounds **11aa**, **11ab**, and **12aa** possessed both the ether (or imino) linker (X = O or NH, see Scheme 2) and the ureido group, and strongly deviated from the others as well as **7ad**. These four compounds more strongly expressed cytotoxic activity against NIH3T3 normal cells than against Sarcoma-180 cancer cells. The cell selectivity, however, was only 2-fold. Furthermore, it is interesting to compare a pair of the *N*-methylureido- and the corresponding *N*-methyl-*N*-nitrosoureido-compounds **7ad** and **8ad** with another pair of the compounds **11aa** and **12aa**. Whereas the introduction of the nitroso group increased the activity of **7ad** against Sarcoma-180, it decreased that of **11aa**. It was highlighted that four derivatives **5ab**, **8aa**, **22**, and **23** exhibited smaller cytotoxic activities against NIH3T3 normal cells than those against Sarcoma-180 cancer cells. Among these four compounds, only 9-hydroxyellipticine (**23)** was uncharged.

As mentioned above, the uncharged derivatives **17aa**, **17ba**, **19aa**, **1**, **23**, and **16a** exhibited extremely potent cytotoxic activity. For this reason, these compounds are the most promising antitumor lead compounds.

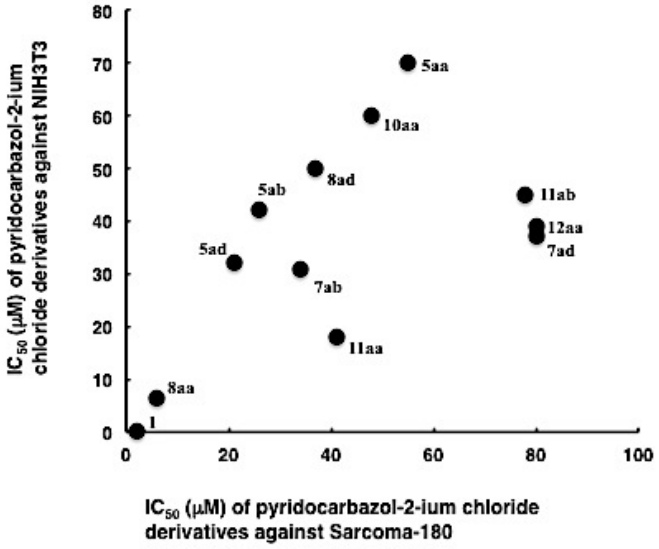


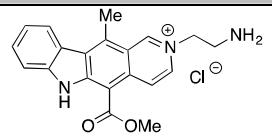
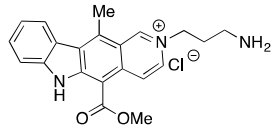
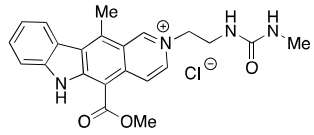
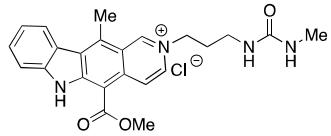
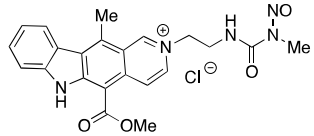
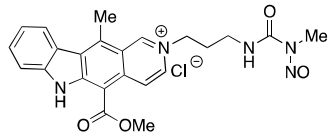
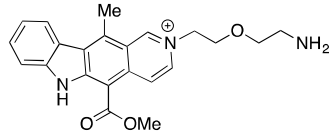
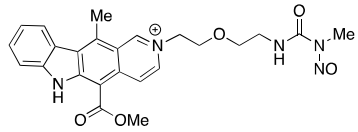
Fig. 5. Relationship of the cytotoxic activity of pyridocarbazol-2-ium chloride derivatives against Sarcoma-180 vs. that against NIH3T3 (y = 1.24x + 0.42, relation coefficient r = 0.967 with the exceptions of **7ad**, **11aa**, **11ab**, and **12aa**).

2.3.2. Effects of the substituting position on the cytotoxic activity

Previously, we reported that 2-alkyl-5-methoxycaronyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride derivatives, which were linked to amine, urea, and nitrosourea moieties via alkyl- and ether-tethers at the N2 atom, exhibited potent cytotoxic activity against HeLa S-3 cells. In order to examine the dependence of the substituting position on the cytotoxic activity, we attempted to compare the cytotoxic activity of the C5-substituted derivatives with that of the compound possessing the same substituent on the N2 position, using HeLa S-3, Sarcoma-180, NIH-3T3, and L1210 cell lines. The previously reported cytotoxic activity[17] of the N2-linked 2-alkyl-5-methoxycaronyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride derivatives **24a**,**b**, **25a**,**b**, **26a**,**b**, **27** and **28** against HeLa S-3 are summarized in Table 3. Most of the IC_{50} values of the C5-linked derivatives against all of the tested cell lines were larger than those of the N2-linked derivatives against HeLa S-3 cells (see Tables 1-3). In other words, most of the N2-linked derivatives exhibited more potent cytotoxic activity than the C5-linked derivatives. The lone exception was a pair of **8aa** and **26a** (Table 1, entry 13 and Table 3, entry 5). In this case, the cytotoxic activity of the C5-linked compound **8aa** against HeLa S-3 cells was slightly more potent than that of the N2-linked compound **26a** (2.5-fold).

Table 3. Cytotoxic activity of 2-alkyl-11-methyl-5-methoxycarbonyl-6*H*-pyrido[4,3-*b*]- calbazol-2-ium chlorides **24**, **25**, **26**, **27** and **28** against the human cervical cancer cell line HeLa S-3[a,b]

Entry	Compound	IC_{50} (μM)

1		**24a**	6 ± 2
2		**24b**	2.50 ± 0.08
3		**25a**	7 ± 2
4		**25b**	24.0 ± 0.6
5		**26a**	15 ± 4
6		**26b**	1.30 ± 0.03
7		**27**	41 ± 2
8		**28**	52 ± 3
9	*cis*-[$PtCl_2(NH_3)_2$]	CDDP	2.0 ± 0.4

[a] Quoted from Ref. 17. [b] MTT assay was used and IC_{50} values are reported as the means of three experiments; the standard deviations are given together.

3. Conclusion

In conclusion, we successfully synthesized 6 types of *N*-methyl-*N*-nitrosourea conjugates of 6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride derivatives and 3 types of the corresponding 6*H*-pyrido[4,3-*b*]carbazole derivatives, which were linked with various types of *N*-alkylcarbamoyl chains on the C-5 atom, and 26 types of the related compounds in good to high yields. Some of these compounds exhibited high cytotoxic activity. The quarternized 5-(*N*-alkyl)carbamyl-2-methylpyridocarbazol-2-ium derivatives with an odd number of methylene units as an *N*-alkylcarbamoyl linker showed higher activity than those with an even number of methylene units. Thus, we found odd-even effect in cytotoxic activity for the first time. On the other hands, the replacement of the central methylene group in the pentamethylene linker unit to the ether (-O-) or imino (-NH-) group suppressed the cytotoxic activity. Furthermore, quarternization of the N-2 atom of the pyridocarbazole ring by the methyl group suppressed the cytotoxic activity by 25- to 96-fold in comparison with the corresponding

un-quarternized pyridocarbazoles. Furthermore, the cytotoxic activities of the amine-, *N*-methylurea-, and *N*-methyl-*N*-nitrosourea-conjugates of 2,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride linked at C5 atom, **5**, **7**, **8**, **10**, and **12**, against Sarcoma-180 exhibited a positive linear relationship with those of the same compounds against NIH3T3. The cytotoxic activities of **5**, **7**, **8**, **10**, and **12** against most of the tested cell lines were less potent than those of the N2-linked conjugates **24**-**28** against HeLa S-3 cells. *N*-(2-Aminoethyl)-11-methyl-6*H*-pyrido[4,3-*b*]carbazole-5-carboxamide (**17aa**), *N*-(2-aminoethyl)-9-methoxy-11-methyl-6*H*-pyrido[4,3-*b*]carbazole-5-carboxamide (**17ba**), and 11-methyl-*N*-(2-(3-methyl-3-nitrosoureido)ethyl)-6*H*-pyrido[4,3-*b*]carbazole-5-carboxamide (**19aa**), ellipticine (**1**), 9-hydroxyellipticine (**23**), methyl 11-methyl-6*H*-pyrido[4,3-*b*]carbazole-5-carboxylate (**16a**) exhibited very potent cytotoxic activity against Sarcoma-180, NIH3T3, and HeLa S-3 cell lines and were the most promising antitumor lead compounds. Furthermore, **19aa**, **1**, **23**, and **16a** exhibited remarkable cell selectivity. However, these compounds, especially **1**, **23**, and **16a**, are sparingly soluble in water.[31] In order to develop water-soluble un-quarternized ellipticine analogues, synthesis and cytotoxic activity of the corresponding PEG ester derivatives of **1** and **16a** are under investigation together with development of the 2-substituted 1,2-dihydroellipticine derivatives as the pro-drugs of ellipticine (**1**). Some of these ellipticine analogues will be applied to evaluation of an *in vivo* antitumor activity, using tumor-bearing mouse, in the near future.

4. Experimental

4.1. Materials and methods

All chemicals were purchased from TCI or Wako and used without further purification, unless otherwise noted. Ellipticine (**1**), 2-methylellipticin-2-ium chloride (**22**), 9-hydroxyellipticine (**23**), 5-methoxycarbonyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazole (**16a**), and 5-methoxycarbonyl-2,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium chloride (**3a**) were synthesized by the reported methods.[17,29] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Nacalai Tesuque or Sigma. Solvents were basically dried and distilled before use. Reaction progress and compound purity were monitored using thin-layer chromatography (TLC) with hexane-ethyl acetate as the irrigating system and UV light at shorter wavelengths as the visualizer. Flash chromatography was performed using silica gel (Merck). Melting points were determined using a Yanaco micro-melting-point apparatus and uncorrected values were reported. ^{1}H and ^{13}C NMR spectra were recorded using a JEOL JNM-ECP500 (500 and 125 MHz) and a JEOL JNM-ECP300 or AL300 (300 and 75 MHz) spectrometers, respectively. The ^{1}H, ^{13}C chemical shifts were referenced to TMS (δ 0.00) using the solvent residual peak as a reference and *J* values are reported in Hz. Mass spectra were recorded on a JEOL JMS-MS700 mass spectrometer using NBA (3-nitrobenzyl alcohol) as a matrix and Xenon (6 kV, 10 mA) as the fast atom bombardment (FAB) gas. FTIR spectra were recorded using a JEOL JIR-5300 or a Thermo Nicolet Continum Microscope IR spectrometer. Fluorescence micrographs were taken on an OLYMPAS CK30 fluorescence microscope equipped with a cooled CCD camera.

Absorbance of MTT formazan was measured on a CORONA MTP-500 microplate reader. CLogP was calculated using ChemBioDraw Ultra software (version 13.0).

4.2. Syntheses

4.2.1. Synthesis of 2,11-dimethyl-5-(ω-aminoalkyl)carbamoyl-6H-pyrido[4,3b]carbazol-2-ium chloride derivatives (pyridocarbazoliums) ***5aa-ad****,* ***5ba, 10aa****, and* ***10ab***

A flask containing a magnetic stirring bar was charged with methyl pyridocarbazole-5-carboxylate **3a** (46 mg, 0.15 mmol) and ethylenediamine (**4a**) (1.38 g, 23 mmol). The reaction mixture was stirred at room temperature (rt) for 3 days. After all the volatile materials were removed under reduced pressure, the crude residue (crude **5aa**) was purified either by ODS column chromatography (H_2O/MeOH = 50 : 1) to yield **5aa** (82%), or by recrystallization from acetonitrile. The compounds **5ab** (83%), **5ac** (91%) and **5ad** (86%) were obtained from the diamines **4b**-**d** in a similar fashion. The reaction of **3b** with **4a** was performed in benzene (1 mL) as a solvent, and the crude product was purified by ODS column chromatography eluting with phosphate buffer (pH 3.3), and further desalted via an Amberlite XAD-2 column to give pure **5ba** in a 62% yield. The reaction of **3a** with the amines **9a**, possessing an ether-group at the central position of a molecule, was performed at 50 ^oC for 3 days leading to the formation of **10aa** in a 60% yield, and the reaction with **9b** was performed under microwave irradiation for 2 h to give **10ab** in a 66% yield.

5aa: yellow solid; decomp. 240 ^oC; IR (KBr): ν/cm^{-1} 3253 (m), 1635 (s), 1597 (s), 1425(s), 1240 (m); ^1H NMR (300 MHz, CD_3OD): δ10.0 (1H, s), 8.49 (2H, m), 8.35 (1H, d, J = 7 Hz), 7.68 (2H, m), 7.47 (1H, m), 4.50 (3H, s), 3.71 (2H, t, J = 12Hz), 3.44 (3H, s), 3.05 (2H, t, J = 12 Hz); MS (FAB): *m/z* 333 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{20}H_{21}N_4O^+$: 333.1716, found: 333.1701.

5ab: yellow solid; decomp. 320 ^oC; IR (KBr): ν/cm^{-1} 3367, 1722; ^1H NMR (500 MHz, D_2O): δ 8.53 (1H, s), 7.66 (1H, d, J = 6.9 Hz), 7.62 (1H, d, J = 6.9 Hz), 7.14 (1H, d, J = 7.3Hz), 6.94 (1H, t, J = 7.3 Hz), 6.73 (1H, d, J = 7.3 Hz), 6.62 (1H, t, J = 7.3 Hz), 3.96 (3H, s), 3.44 (2H, t, J = 7.1 Hz), 2.97 (2H, t, J = 7.6 Hz), 2.10 (3H, s), 1.29 (2H, m); MS (FAB^+): *m/z* 347 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{21}H_{23}N_4O^+$: 347.1872, found: 347.1869.

5ac: yellow solid; decomp. 215 ^oC; ^1H NMR (500 MHz, D_2O): δ8.39 (1H, s), 7.60 (1H, d, J = 7.5 Hz), 7.51 (1H, d, J = 7.5 Hz), 6.39 (1H, d, J = 6.0 Hz), 6.83 (1H, m), 6.58 (1H, d, J = 6.0 Hz), 6.49 (1H, t, J = 6.0 Hz), 3.92 (3H, s), 3.37 (2H, t, J = 7.0 Hz), 2.99 (2H, t, J = 7.0 Hz), 1.88 (3H, m), 1.66 (4H, m); ^{13}C NMR (125 MHz, CD_3OD): δ167.2, 148.0, 144.1, 143.9, 138.1, 134.0, 133.0 130.3, 130.2, 125.6, 125.5, 122.7, 122.1, 121.2, 113.0, 111.8, 47.8, 40.7, 40.5, 27.3, 26.3,15.7; MS (FAB): *m/z* 361 (M^+ - Cl^-); HRMS (FAB) Calcd for $C_{22}H_{24}N_4O^+$: 361.2028, found: 361.2031.

5ad: yellow solid; decomp. 210 ^oC; ^1H NMR (500 MHz, D_2O): δ8.52 (1H, s), 7.55 (1H, d, J = 6.5 Hz), 7.51 (1H, d, J = 6.5 Hz), 7.11 (1H, m), 6.95 (1H, t, J = 7.5 Hz), 6.69 (1H, d, J = 7.5 Hz), 6.29 (1H, t, J = 7.5 Hz), 3.97 (3H, s), 3.36 (2H, m), 2.94 (2H, t, J = 7.5 Hz), 2.02 (3H, s), 1.66 (4H, m), 1.38 (2H, t, J = 7.5 Hz); ^{13}C NMR (125 MHz, CD_3OD): δ167.2, 148.0, 144.1, 143.9, 138.0, 134.0, 133.0, 130.3,

128.6, 125.4, 123.0, 122.7, 122.0, 121.2, 112.9, 112.0, 47.8, 41.1, 40.7, 29.8, 28.2, 25.1, 15.7; MS (FAB): *m/z* 375 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{23}H_{27}N_4O^+$: 375.2185, found 375.2193.

5ba: orange solid; decomp. 280 °C; ^{1}H NMR (500 MHz, D_2O): δ 8.92 (1H, s), 7.92 (2H, d, *J* = 7.2 Hz), 7.88 (1H, d, *J* = 7.2 Hz), 6.84 (1H, d, *J* = 8.7 Hz), 6.54 (1H, s), 6.59 (1H, d, *J* = 8.7 Hz), 4.17 (3H, s), 3.74 (2H, t, *J* = 6.6. Hz), 3.55 (3H, s), 3.25 (2H, t, *J* = 6.6. Hz), 2.29 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ 167.6, 153.7, 145.7, 142.6, 137.6, 136.5, 133.0, 131.4, 126.3, 121.4, 120.9, 119.6, 116.6, 112.1, 108.3, 107.4, 56.1, 47.1, 39.2, 38.2, 14.7; MS (FAB): *m/z* 363 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{21}H_{23}N_4O_2^+$: 363.1821, found: 363.1822.

10aa: yellow solid; decomp. 271 °C; ^{1}H NMR (500 MHz, D_2O): δ 8.88 (1H, s), 7.81 (2H, s), 7.54 (1H, d, *J* = 7.5 Hz), 7.20 (1H, t, *J* = 7.0, 7.5 Hz), 6.99 (1H, d, *J* = 8.0 Hz), 6.92 (1H, t, *J* = 7.0, 8.0 Hz), 4.11 (3H, s), 3.67 (6H, m), 3.07 (2H, t, *J* = 5 Hz), 2.45 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ 148.1, 144.1, 143.9, 138.1, 134.1, 133.0, 130.3, 130.2, 167.2, 125.5, 125.4, 122.7, 122.1, 121.2, 113.0, 111.8, 47.8, 40.7, 40.5, 27.3, 26.3, 15.7; MS (FAB): *m/z* 377 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{22}H_{25}N_4O_2^+$: 377.1987, Found: 377.1980

10ab: yellow solid; decomp. 220 °C; ^{1}H NMR (500 MHz, D_2O): δ 8.65 (1H, s), 7.88 (1H, d, *J* = 7.5 Hz), 7.75 (1H, d, *J* = 7.5 Hz), 7.25 (1H, m), 7.01 (1H, t, *J* = 6.5 Hz), 6.86 (1H, t, *J* = 6.5 Hz), 6.69 (1H, d, *J* = 6.5 Hz), 4.05 (3H, s), 3.82 (2H, t, *J* = 6.5 Hz), 3.37-3.48 (6H, m), 2.17 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ 168.3, 146.8, 143.3, 143.2, 138.0, 133.8, 132.7, 129.8, 127.7, 124.9, 122.3, 122.2, 122.1, 120.7, 112.7, 109.7, 47.8, 46.1, 38.4, 38.3, 37.1, 15.7; MS (FAB): *m/z* 376 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{22}H_{26}N_5O^+$: 376.2137, found: 376.2132.

*4.2.2. Synthesis of 2,11-dimethyl-5-(2-(3-methylureido)ethyl)carbamoyl-6H-pyrido[4,3-b]carbazol-2-ium chloride (**7aa**)*

2,5-Dioxopyrrolidin-1-yl *N*-methylcarbamate (22.4 mg, 0.087 mmol) was placed in a reaction flask and dissolved in 3 mL of DMF. After being cooled at -10 °C, a solution of **5aa** (50 mg, 0.072 mmol) and diisopropylethylamine (DIPEA, 53.4 mg, 0.276 mmol) in DMF (2 mL) was added drop-by-drop through a Teflon cannula under an argon atmosphere. The reaction mixture was stirred at -10 to -5 °C for 7 h. After the volatile materials were removed under reduced pressure, the residue was dissolved in a small amount of MeOH. To this solution, ethyl acetate was gradually added to re-precipitate the product, which was collected by filtration using a membrane filter (PTFE 0.5 μm) to give **7aa** (71 %) as a yellow solid; indefinite melting; ^{1}H NMR (300 MHz, CD_3OD): δ 10.1 (1H, s), 8.56 (2H, m), 8.43 (1H, d, *J* = 1.5 Hz), 7.92 (1H, m), 7.78 (1H, m), 7.55 (1H, m), 4.59 (3H, s), 3.74 (2H, t, *J* = 3.0 Hz), 3.66 (2H, t, *J* = 3.0 Hz), 3.51 (3H, s), 2.82(3H, s); MS (FAB): *m/z* 390 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{22}H_{24}N_5O_2^+$: 390.4665, Found: 390.4672.

4.2.3. Synthesis of 2,11-dimethyl-5-(ω-(3-methylureido)alkyl)carbamoyl-6H-pyrido[4,3-b]carbazol-2-

ium chloride derivatives (methylureidopyridocarbazoliums) ***7ab-ad, 7ba***, ***11aa,*** *and* ***11ab***

4-Nitrophenyl *N*-methylcarbamate (**6a**) (22.4 mg, 0.087 mmol) was placed in a reaction flask and dissolved in 3 mL of DMF. After being cooled at -10 ^{o}C, a solution of **5ab** (26 mg, 0.072 mmol) and DIPEA (53.4 mg, 0.276 mmol) in DMF (2 mL) was added drop-by-drop through a Teflon cannula under an argon atmosphere. The reaction mixture was stirred at -10 to -5 ^{o}C for 7 h. After the volatile materials were removed under reduced pressure, the residue was dissolved in a small amount of MeOH. To this solution, ethyl acetate was gradually added to re-precipitate the product, which was collected by filtration using a membrane filter (PTFE 0.5 μm) to give **7ab** (85%). The compounds **7ac** (70%), **7ad** (88%), **7ba** (60%), and **11aa** (70%) were obtained in a similar manner from the pyridocarbazoles **5ac**, **5ad**, **5ba**, and **10aa**. The reaction of **10ab** was performed at -60 ^{o}C for 11 h to give **11ab** in a 56% yield.

7ab: yellow solid; decomp. 280 ^{o}C; ^{1}H NMR (500 MHz, CD_3OD): δ 9.84 (1H, s), 8.31-8.33 (2H, m), 8.25 (1H, m), 7.68 (1H, d, J = 7.5 Hz), 7.62-7.64 (1H, m), 7.39-7.43 (1H, m), 4.47 (3H, s), 3.65 (2H, t, J = 6.5 Hz), 3.31 (2H, t, J = 5.0 Hz), 3.27 (3H, s), 2.74 (3H, s), 1.91 (2H, m); ^{13}C NMR (125 MHz, CD_3OD): δ 174.6, 167.3, 161.5, 147.9, 144.0, 143.8, 138.0, 134.0, 132.9, 130.2, 128.4, 122.6, 122.1, 121.1, 113.0, 111.6, 48.5, 39.5, 38.5, 30.5, 29.7, 27.9, 15.8; MS (FAB$^+$): *m/z* 404 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{23}H_{26}N_5O_2^+$: 404.2086, found: 404.2098.

7ac: yellow solid; decomp. 200 ^{o}C; ^{1}H NMR (500 MHz, D_2O): δ 8.23 (1H, s), 7.45 (1H, d, J = 6.5 Hz), 7.32 (1H, d, J = 6.5 Hz), 6.83 (1H, d, J = 7.0 Hz), 6.70 (1H, t, J = 7.0 Hz), 6.39 (2H, m), 3.75 (3H, s), 3.22 (2H, t, J = 5.5 Hz), 2.96 (2H, t, J = 5.5 Hz), 2.47 (3H, s), 1.47 (3H,s), 1.36-1.47 (4H, m); ^{13}C NMR (125 MHz, CD_3OD): δ 167.1, 161.6, 148.0, 144.1, 143.8. 138.0, 133.9, 132.9, 130.3, 128.6, 125.4, 122.9, 122.7, 122.0, 121.2, 112.9, 112.0, 47.8, 41.2, 41.0, 28.7, 27.6, 27.4, 15.7; MS (FAB): *m/z* 418 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{24}H_{28}N_5O_2^+$: 418.2243, found: 418.2246.

7ad: yellow solid; decomp. 195 ^{o}C; ^{1}H NMR (500 MHz, D_2O): δ 8.52 (1H, s), 7.363 (m), 7.22 (1H, m), 6.67 (1H, t, J = 6.5 Hz), 6.55 (1H, d, J = 6.5 Hz), 6.22 (2H, m), 3.70 (3H, s), 3.10 (2H, m), 2.85 (2H, t, J = 6.5 Hz), 2.39 (3H, s), 1.60 (3H, s), 1.37 and 1.26 (4H, m), 1.14 (2H, m); ^{13}C NMR (125 MHz, CD_3OD): δ 167.2, 161.1, 148.0, 144.0, 143.8, 138.0, 133.9, 132.9, 130.2, 128.5, 125.4, 122.9, 122.6, 122.0, 121.2, 112.9, 112.0, 47.8, 42.1, 41.2, 30.1, 29.9, 28.0, 25,3, 15.7; MS (FAB): 432 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{25}H_{30}N_5O_2^+$: 432.2400, found: 432.2405.

7ba: orange solid; decomp. 280 ^{o}C; ^{1}H NMR (300 MHz, D_2O): δ8.47 (1H, s), 7.61 (1H, d, J = 6.9 Hz), 7.48 (1H, d, J = 6.9 Hz), 6.31 (1H, d, J = 8.4 Hz), 6.06 (1H, d, J = 8.4 Hz), 5.88 (1H, s), 3.90 (3H, s), 3.36 (2H, m), 3.20 (5H, m), 2.50 (3H, s), 1.66 (3H, s); ^{13}C NMR (125 MHz, D_2O): δ167.7, 162.6, 156.6, 148.2, 144.1, 138.7, 138.4, 134.0, 133.0, 128.6, 123.6, 122.3, 121.0, 118.5, 113.9, 111.7, 109.1, 56.8, 55.1, 42.6, 41.3, 27.9, 15.9; MS (FAB): *m/z* 420 (M^+- Cl^-); HRMS (FAB): Calcd for $C_{23}H_{26}N_5O_2^+$: 420.2036, found: 420.2008.

11aa: yellow solid; decomp. 223 ^{o}C; ^{1}H NMR (500 MHz, CD_3OD): δ9.75 (1H, s), 8.28 (2H, m), 8.22 (1H, d, J = 7.5 Hz), 7.56 (2H, m), 7.34 (1H, d, J = 7.5 Hz), 4.40 (3H, s), 3.76 (3H, s), 3.57 (2H,

d), 3.17 (2H, d), 2.92 (2H, d), 2.78 (2H, d), 2.50 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ 167.2, 161.7, 148.1, 144.1, 143.9, 138.0, 133.9, 132.9, 130.3, 128.6, 125.5, 122.9, 122.7, 122.2, 121.2, 113.0, 112.1, 47.9, 41.3, 41.0, 28.8, 27.6, 27.5, 15.7; MS (FAB): *m/z* 435 (M^+-Cl^-); HRMS (FAB): Calcd for $C_{24}H_{28}N_5O_3^+$: 434.2192, found: 434.2191.

11ab: yellow solid; decomp. 215 ^{o}C; ^{1}H NMR (500 MHz, D_2O): δ 8.90 (1H, s), 7.94 (1H, d, *J* = 6.5 Hz), 7.83 (1H, d, *J* = 6.5 Hz), 7.52 (1H, m), 7.13 (1H, m), 6.97 (1H, m), 6.87 (1H, m), 4.13 (3H, s), 3.80 (2H, t, *J* = 6.0 Hz), 3.15-3.36 (6H, m), 2.44 (3H, s), 2.33 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ 168.5, 162.4, 147.2, 143.8, 143.6, 138.3, 134.0, 132.9, 130.0, 127.9, 125.2, 122.5, 122.4, 121.2, 120.9, 112.9, 110.0, 50.6, 47.8, 45.9, 38.3, 38.0, 27.2, 15.8; MS (FAB): *m/z* 433 (M+ - Cl-); HRMS (FAB): Calcd for $C_{24}H_{29}N^6O_2^+$: 433.2352, found: 433.2359.

*4.2.4. Synthesis of 2,11-dimethyl-5-(2-(3-methyl-3-nitrosoureido)ethyl)carbamoyl-6H-pyrido[4,3-b]carbazol-2-ium chloride (**8aa**)*

2,5-Dioxopyrrolidin-1-yl *N*-methyl-*N*-carbamate (17.5 mg, 0.087 mmol) was placed in a reaction flask and dissolved in 3 mL of DMF. After being cooled at -10 ^{o}C, a solution of **5aa** (50 mg, 0.072 mmol) and DIPEA (53.4 mg, 0.276 mmol) in DMF (2 mL) was added drop-by-drop through a Teflon cannula under an argon atmosphere. The reaction mixture was stirred at -10 to -5 ^{o}C for 7 h. After the volatile materials were removed under reduced pressure, the residue was dissolved in a small amount of MeOH. To this solution, ethyl acetate was gradually added to re-precipitate the product, which was collected by filtration using a membrane filter (PTFE 0.5 μm) to give **8aa** (76%) as yellow solid; indefinite melting; ^{1}H NMR (300 MHz, CD_3OD) δ 9.93 (1H, s), 8.44 (2H, m), 8.24 (1H, d), 7.65(2H, m), 7.41 (1H, t), 4.44 (3H, s), 3.78 (3H, s), 3.36 (4H, s), 3.18 (3H, s); MS (FAB^+): *m/z* 419 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{22}H_{23}N_6O_3^+$: 419.4645, Found: 419.4652.

*4.2.5. Synthesis of 2,11-dimethyl-5-(ω-(3-methyl-3nitrosoureido)alkylcarbamoyl)-6H-pyrido[4,3-b]carbazol-2-ium chloride derivatives (methylnitrosoureidopyridocarbazolium) **8ab-ad, 8ba** and **12aa***

4-Nitrophenyl *N*-methyl-*N*-nitrosocarbamate (**6b**) (15.5 mg, 0.069 mmol) was placed in a reaction flask and dissolved in 3 mL of DMF. After being cooled at -10 ^{o}C, a solution of **5ab** (20.6 mg, 0.057 mmol) and DIPEA (29.0 mg, 0.15 mmol) in DMF (2 mL) was added drop-by-drop through a Teflon cannula under an argon atmosphere. The reaction mixture was stirred at -10 to -5 ^{o}C for 7 h. After the volatile materials were removed under reduced pressure, the residue was dissolved in a small amount of MeOH. To this solution, ethyl acetate was gradually added to re-precipitate the product, which was collected by filtration using a membrane filter (PTFE 0.5 μm) to give **8ab** (82 %). The compounds **8ac** (70%), **8ad** (77%), **8ba** (45%), **12aa** (63%) were obtained in a similar manner from the pyridocarbazoles **5ac**, **5ad**, **5ba**, and **10aa**.

8ab: yellow solid; decomp. 225 ^{o}C; ^{1}H NMR (500 MHz, CD_3OD): δ 9.76 (s, 1H), 8.19-8.29 (2H, m), 8.17 (1H, d, *J* = 8.25 Hz), 7.52-7.56 (2H, m), 7.30-7.35 (1H, m), 4.38 (3H, s), 3.62 (2H, t, *J* = 6.9

Hz), 3.54 (2H, t, *J* = 6.5 Hz), 3.19 (3H, s), 3.10 (3H, s), 1.97 (2H, m); MS (FAB$^+$): *m/z* 433 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{23}H_{25}N_6O_3^+$: 433.1989, found: 433.1995.

8ac: yellow solid; decomp. 145 ^oC; ^1H NMR (500 MHz, D_2O): δ 8.53 (1H, s), 7.61 (1H, d, *J* = 7.0 Hz), 7.54 (1H, d, *J* = 7.0 Hz), 7.13 (1H, d, *J* = 7.5 Hz), 6.92 (1H, m), 6.65 (2H, m), 3.96 (3H, s), 3.36 (4H, m), 2.97 (3H, s), 2.09 (3H, s), 1.61 (4H, m); ^{13}C NMR (125MHz, $CDCl_3$): δ 169.5, 162.0, 155.2, 150.2, 144.4, 141.3, 134.6, 133.9, 133.1, 128.9, 126.5, 125.0, 124.1, 123.1, 121.2, 118.6, 112.2, 111.0, 40.9, 40.8, 29.1, 27.8, 27.0, 15.2; MS (FAB): *m/z* 447 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{24}H_{27}N_6O_3^+$: 447.2145, found: 447.2138.

8ad: yellow solid; decomp. 142 ^oC; ^1H NMR (500 MHz, D_2O): δ 8.60 (1H, s), 7.65 (1H, d, *J* = 6.5 Hz), 7.58 (1H, d, *J* = 6.5 Hz), 7.26 (1H, d, *J* = 7.5 Hz), 6.97 (1H, d, *J* = 7.5 Hz), 6.71 (2H, m), 3.99 (3H, s), 3.33 (2H, m), 3.28 (2H, t, *J* = 6.5 Hz), 2.48 (4H, m), 2.16 (3H, s), 1.57 (5H, m); MS (FAB): *m/z* 461(M^+ - Cl^-), HRMS (FAB): Calcd for $C_{25}H_{29}N_6O_3^+$: 461.2301, found 461.2295.

8ba: orange solid; decomp. 275 ^0C; ^1H NMR (300 MHz, D_2O): δ 8.84 (1H, s), 7.77 (2H, m), 6.73 (1H, d, *J* = 5.1 Hz), 6.31 (1H, d, *J* = 8.4 Hz), 6.52 (2H, m), 4.12 (3H, s), 3.65 (4H, m), 3.51 (3H, s), 3.00 (3H, s), 2.12 (3H, s); MS (FAB): *m/z* 449 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{23}H_{25}N_6O_4^+$: 449.1937, found: 449.1930.

12aa: yellow solid; decomp. 271 ^0C; ^1H NMR (500 MHz, CD_3OD): δ 9.95 (1H, s), 8.43 (2H, m), 8.33 (1H, d, *J* = 8.0 Hz), 7.66 (2H, m), 7.44 (1H, dd, *J* = 7.0, 7.5 Hz), 4.50 (3H, s), 3.84 (4H, m), 3.71 (2H, t, *J* = 5.5 Hz), 3.64 (2H, t, *J* = 5.5 Hz), 3.37 (3H, s), 2.00 (3H, s); MS (FAB): *m/z* 463 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{24}H_{27}N_6O_4^+$: 463.2094, found: 463.2101.

4.2.6. Synthesis of 11-methyl-5-(ω-aminoalkyl)carbamoyl-6H-pyrido[4,3b]carbazole derivatives (aminoalkylpyridocarbazoles) ***17aa-ac*** *and* ***17ba***

Methyl 11-methylpyrido[4,3-*b*]carbazole-5-carboxylate (**16a**) (20 mg, 0.086 mmol) was added to ethylenediamine (0.78 g, 12.9 mmol), and the reaction mixture was stirred at rt for 3 days. The reaction mixture was evaporated to dryness under reduced pressure. The residue was purified by silica gel chromatography ($CHCl_3$: MeOH = 60 :1) to give **17aa** (33 mg, 72%). The compounds **17ab** (70%), **17ac** (73%), and **17ba** (65%) were obtained in a similar manner from the pyridocarbazoles **16a** and **16b**, respectively.

17aa: yellow solid; decomp. 227.6 ^oC; ^1H NMR (500MHz, $CDCl_3$): δ 11.04 (1H, s), 9.40 (1H, s), 8.34 (1H, d, *J* = 6.0 Hz), 8.10 (1H, d, *J* = 7.5 Hz), 7.93 (1H, d, *J* = 6.0 Hz), 7.41 (1H, t, *J* = 7.5 Hz), 7.23 (1H, d, *J* = 7.5 Hz), 6.73 (1H, t, *J* = 7.5 Hz), 3.59 (2H, t, *J* = 5.7 Hz), 3.01 (2H, t, *J* = 5.7 Hz), 2.91 (3H, s), 1.2 (2H, br); ^{13}C NMR (125MHz, $CDCl_3$): δ 165.1, 148.3, 141.6, 140.2, 138.3, 131.1, 129.7, 126.2, 122.8, 122.5, 121.1, 120.1, 118.3, 115.7, 109.9, 109.3, 40.5, 39.4, 13.5; MS (FAB): *m/z* 319 (M + H); HRMS (FAB): Calcd for $C_{19}H_{19}N_4O^+$: 319.1559, Found: 319.1562

17ab: yellow solid; indefinite melting; ^1H NMR (500MHz, $CDCl_3$): δ 10.43 (1H, s), 9.74 (1H, s), 8.53 (1H, d, *J* = 6 Hz), 8.36 (1H, d, *J* = 8.0 Hz), 8.21 (1H, d, *J* = 6 Hz), 7.55 (1H, m), 7.47 (1H, d, *J*

=8.0 Hz), 7.34 (1H, m), 3.81 (2H, q, *J* = 5.5 Hz), 3.34 (3H, s), 3.01 (2H, t, *J* = 5.5 Hz), 1.88 (2H, t, *J* = 6.0 Hz), 1.27 (2H, br); ^{13}C NMR (125MHz, $CDCl_3$): δ167.6, 150.1, 142.5, 142.2, 134.6, 131.6, 127.7, 125.1, 124.0, 123.1, 120.3, 116.6, 110.9, 107.0, 77.2, 41.0, 39.6, 31.5, 15.3, 14.1; MS (FAB): *m/z* 333 (M + H); HRMS (FAB): Calcd for $C_{20}H_{21}N_4O^+$: 333.1715, found 333.1693.

17ac: yellow solid; indefinite melting; ^{1}H NMR (500MHz, CD_3OD): δ9.55 (1H, s), 8.32 (2H, m), 7.96 (1H, d, *J* = 4.5 Hz), 7.53 (2H, m), 7.28 (1H, t, *J* = 7 Hz), 3.61 (2H, t, *J* = 7 Hz), 3.22 (3H, s), 2.73-2.76 (2H, t, *J* = 7 Hz), 1.78 (2H, quintet, *J* = 7.5 Hz), 1.64 (2H, quintet, *J* = 7.5 Hz); ^{13}C NMR (125MHz, $CDCl_3$): δ169.4, 150.1, 144.3, 141.3, 134.6, 133.0, 128.8, 126.4, 124.9, 124.0, 123.0, 121.2, 118.6, 112.1, 110.8, 42.2, 41.1, 31.2, 28.3, 27.9, 15.2; MS (FAB): *m/z* 347 (M + H); HRMS (FAB): Calcd for $C_{21}H_{23}N_4O$: 347.1872, found: 347.1876.

17ba: yellow solid; decomp. 249 ^{o}C; ^{1}H NMR (500MHz, CD_3OD): δ 9.88 (1H, s), 8.53 (1H, d, *J* = 6.5 Hz), 8.34 (1H, d, *J* = 7.0 Hz), 7.90 (1H, s), 7.62 (1H, d, *J* = 7.0 Hz), 7.29 (1H, d, *J* = 6.5 Hz), 3.96 (3H, s), 3.91 (2H, t, *J* = 6.0 Hz), 3.01 (2H, t, *J* = 6.0 Hz), 3.36 (3H, s); ^{13}C NMR (125MHz, DMSO-d_6): δ165.5, 154.3, 144.2, 142.2, 137.4, 137.2, 132.7, 129.2, 126.5, 122.1, 120.4, 119.1, 117.0, 112.8, 110.7, 107.9, 55.8, 38.3, 37.7, 15.4; MS (FAB): *m/z* 349 (M+H); HRMS (FAB): Calcd for $C_{20}H_{21}O_2N_4^+$: 349.1665, found: 349.1678.

4.2.7. Synthesis of 11-methyl-5-(ω-(3-methylureido)alkyl)carbamoyl-6H-pyrido[4,3b]carbazole derivatives (methylureidopyridocarbazoles) ***18aa-ac*** *and* ***18ba***

4-Nitrophenyl *N*-methylcarbamate (**6a**) (24 mg, 0.13 mmol) and DIPEA (50 mg, 0.39 mmol) were placed in a reaction flask and dissolved in 4 mL of DMF. After being cooled at -10 ^{o}C, a solution of **17aa** (33 mg, 0.10 mmol) in DMF (3 mL) was added drop-by-drop through a Teflon cannula under an argon atmosphere. The reaction mixture was stirred at -10 to -5 ^{o}C for 7 h. After the volatile materials were removed under reduced pressure, the residue was purified by amino-silica gel chromatography ($CHCl_3$: CH_3OH = 9 : 1) to give **18aa** (82%). The compounds **18ab** (80%), **18ac** (80%), and **18ba** (79%) were obtained in a similar manner from the pyridocarbazoles **17ab**, **17ac**, **5ba**, and **17ba**, respectively.

18aa: yellow solid; decomp; 250.4 ^{o}C; ^{1}H NMR (500 MHz, CD_3OD): δ9.53 (1H, s), 8.29 (1H, d, *J* = 13.0 Hz), 8.24 (d, 1H, *J* = 10.0 Hz), 7.92 (1H, d, *J* = 10.0 Hz), 7.57 (1H, d, *J* = 13.5 Hz), 7.44 (1H, t, *J* = 12.5, 13.5 Hz), 7.21 (1H, t, *J* = 12.5, 13.0 Hz), 3.57 (t, 2H, *J* = 8.5 Hz), 3.45 (t, 2H, *J* = 8.5 Hz), 3.23 (3H, s), 2.63 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6): δ166.2, 159.5, 149.5, 142.8, 141.4, 138.7, 132.2, 130.9, 127.4, 124.0, 123.7, 122.3, 121.3, 119.6, 117.0, 111.2, 110.5, 40.8, 40.1, 26.6, 14.7; MS (FAB): *m/z* 376 ($M+H^-$); Calcd for $C_{21}H_{22}N_5O_2^+$: 376.1774, found: 376.1775.

18ab: yellow solid; indefinite melting; ^{1}H NMR (500 MHz, CD_3OD): δ9.54 (1H, s), 8.29 (2H, m), 7.96 (1H, d, *J* = 4.5 Hz), 7.57 (1H, d, *J* = 8 Hz), 7.49 (1H, t, *J* = 8 Hz), 7.27 (1H, t, *J* = 7.5 Hz), 3.64 (1H, q, *J* = 7 Hz), 3.33 (3H, s), 3.20 (2H, m), 2.72 (3H, s), 1.90 (2H, t, *J* = 6.5 Hz); ^{13}C NMR (125

MHz, CD_3OD): δ 169.6, 162.0, 150.0, 144.3, 141.2, 140.2, 134.6, 133.0, 128.8, 127.2, 126.4, 124.0, 123.0, 121.1, 118.6, 112.2, 110.7, 38.5, 38.4, 31.1, 27.1, 15.2: MS (FAB): *m/z* 391 (M + H); HRMS (FAB): Calcd for $C_{22}H_{24}N_5O_2^+$: 390.1930, found: 390.1929.

18ac: yellow solid; indefinite melting; ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.64 (1H, s), 8.40 (1H, d, *J* = 8.5 Hz), 8.37 (1H, d, *J* = 6.0 Hz), 8.00 (1H, d, *J* = 6.0 H), 7.59 (1H, d, *J* = 8.0 Hz), 7.54 (1H, m), 7.32 (1H, m), 3.62 (2H, t, *J* = 7 Hz), 3.31 (3H, s), 3.21 (2H, t, *J* = 7 Hz), 2.68 (3H, s), 1.78 (2H, quintet, *J* = 7 Hz), 1.66 (2H, quintet, *J* = 7 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.5, 162.0, 155.2, 150.2, 144.4, 141.3, 134.6, 133.1, 128.9, 126.5, 125.0, 124.1, 123.1, 121.2, 118.6, 112.2, 111.0, 40.9, 40.8, 29.1, 27.8, 27.0, 15.2; MS (FAB): *m/z* 404 (M + H); HRMS (FAB): Calcd for $C_{23}H_{25}N_5O_2^+$: 404.2087, found: 404.2094.

18ba: yellow solid; decomp; 267.8 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.25 (1H, s), 9.71 (1H, s), 8.77 (1H, s), 8.38 (1H, d, *J* = 6.0 Hz), 7.90 (2H, m), 7.61 (1H, d, *J* = 6.5 Hz), 7.21 (1H, d, *J* = 6.5 Hz), 6.26 (1H, s-like), 6.03(1H, s-like), 3.90 (3H, s), 3.45 (2H, t, *J* = 5.5 Hz), 3.37 (2H, t, *J* = 5.5 Hz), 3.32 (3H, s), 2.60 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6): δ 166.2, 159.5, 153.3, 149.6, 141.3, 139.3, 137.3, 132.4, 130.9, 124.1, 122.8, 121.0, 117.0, 115.5, 111.6, 110.2, 107.7, 55.8, 40.7, 40.0, 26.5, 14.6; MS (FAB): *m/z* 406 (M+H); HRMS (FAB): Calcd for $C_{22}H_{24}N_5O_3^+$: 406.1879, found: 406.1884.

*4.2.8. Synthesis of 11-methyl-5-(2-(3-methyl-3-nitrosoureido)ethyl)carbamoyl-6H-pyrido[4,3b]-carbazole (**19aa**) and its 9-methoxy derivative **19ba***

4-Nitrophenyl *N*-methylcarbamate (**6a**) (37 mg, 0.16 mmol) and DIPEA (67 mg, 0.51 mmol) were placed in a reaction flask and dissolved in 4 mL of DMF. After being cooled at -10 ^{o}C, a solution of **17aa** (43 mg, 0.14 mmol) in DMF (3 mL) was added drop-by-drop through a Teflon cannula under an argon atmosphere. The reaction mixture was stirred at -10 to -5 ^{o}C for 7 h. After the volatile materials were removed under reduced pressure, the residue was purified by silica gel chromatography ($CHCl_3$: CH_3OH = 5 : 1) to give **19aa** (70 %). The compounds **19ba** was obtained in a similar manner from the pyridocarbazoles **17ba** in a 60% yield.

19aa: yellow solid; decomp. 230.7 ^{o}C; ^{1}H-NMR (500MHz, DMSO-d_6) δ 11.25 (1H, s), 9.73 (1H, s), 8.93 (1H, t, *J* = 5.0 Hz), 8.82 (1H, t, *J* = 5.0 Hz), 8.41 (1H, d, *J* = 7.5 Hz), 8.38 (1H, *J* = 5.5 Hz), 7.95 (1H, d, *J* = 6.5 Hz), 7.63 (1H, d, *J* = 7.5 Hz), 7.54 (1H, t, *J* = 7.5 Hz), 7.29 (1H, t, *J* = 7.5 Hz), 3.69 (3H, s), 3.33 (3H, s), 3.15 (4H, m); ^{13}C NMR (125 MHz, DMSO-d_6): δ 165.8, 154.2, 149.5, 142.6, 141.3, 138.4, 131.9, 130.9, 127.4, 123.9, 123.7, 122.4, 121.2, 119.6, 116.6, 110.8, 110.0, 48.57, 42.3, 36.0, 14.7; MS (FAB): *m/z* 405 (M+H); HRMS (FAB): Calcd for $C_{21}H_{21}N_6O_3^+$: 405.1675, Found: 405.1672.

19ba: yellow solid; decomp. 284 ^{o}C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.05 (1H, s), 9.72 (1H, s), 8.92 (1H, t, *J* = 5.5 Hz), 8.79 (1H, t, *J* = 5.5 Hz), 8.35 (1H, d, *J* = 6.0 Hz), 7.93 (1H, d, *J* = 6.0 Hz), 7.88 (1H, s), 7.55 (1H, d, *J* = 8.5 Hz), 7.20 (1H, d, *J* = 8.5 Hz), 3.90 (3H, s), 3.67 (4H, m), 3.33 (3H, s),

3.12 (3H, s); MS (FAB): *m/z* 435 (M+H); HRMS (FAB): Calcd for $C_{22}H_{23}N_6O_4^+$: 435.1781, found: 435.1772.

*4.2.9. Synthesis of 11-methyl-5-(3-(3-methyl-3-nitrosoureido)propyl)carbamoyl-6H-pyrido[4,3b]-carbazole (**19ab**) by nitrosation of **18ab***

The *N*-methylurea **18ab** (38.9 mg, 0.10 mmol) was placed in a reaction flask and dissolved in hydrochloric acid, and then cooled at 0 ^oC in an ice-bath. To the solution was added an aqueous solution of sodium nitrite (104 mg, 1.5 mmol) drop by drop, and the mixture was stirred at 0 ^oC for 1 h. After the reaction, the mixture was neutralized with 20% aqueous sodium carbonate, and then a precipitated product was filtrated, followed by washing with cold water, to obtain the nitrosated derivative **19ab** in an 80% yield as a yellow solid; indefinite melting; ^{1}H-NMR (500MHz, DMSO-d_6) δ 11.38 (1H, s), 9.76 (1H, s), 8.87 (1H, t, *J* = 6 Hz), 8.74 (1H, t, *J* = 6 Hz), 8.43 (1H, d, *J* = 6 Hz), 8.41 (d, *J* = 7.5 Hz), 7.96 (1H, d, *J* = 6 Hz), 7.65 (1H, d, *J* = 7.5 Hz), 7.54 (1H, t, *J* = 7.5 Hz), 7.29 (1H, t, *J* = 7.5 H), 3.53 (2H, t, *J* = 6.5 Hz), 3.45 (2H, t, *J* = 6.5 Hz), 3.39 (3H, s), 3.10 (3H, s), 1.95 (2H, t, *J* = 6.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 166.0, 153.0, 149.2, 142.7, 140.6, 139.1, 132.5, 130.9, 127.5, 124.2, 123.7, 122.3, 119.7, 117.1, 115.8, 111.3, 110.5, 39.0, 38.2, 29.0, 27.0, 14.8; MS (FAB): *m/z* 419 (M + H); HRMS (FAB): Calcd for $C_{22}H_{23}N_6O_3^+$: 419.1832, found: 419.1831.

*4.2.10. Synthesis of amine conjugates of pyridocarbazole derivatives with a peptide linker, **20a**,**b***

A mixture of 9-fluorenylmethoxycarbonylglycine (Fmoc-glycine) (18 mg, 0.061 mmol), *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate (TBTU) (15 mg, 0,046 mmol), 1-hydroxybenzotriazole (HOBt) (7 mg, 0.046 mmol), and DIPEA (10 mg, 0.075 mmol) in DMF (2 mL) was stirred at rt for 1 h. The solution was added to a solution of 5-((2-aminoethyl)carbamoyl)pyridocarbazole **5aa** (15 mg, 0.041 mmol) in DMF (1 mL), and stirred at 60 ^oC for 2 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by ODS chromatography (H_2O-CH_3OH) to give the corresponding Fmoc-protected product, to which piperidine (0.25 mL) was added in DMF (1 mL). After stirring at rt for 1 h, the reaction mixture was concentrated under reduced pressure, purified by ODS chromatography (phosphate buffer: pH 3.3), and successively desalted via an Amberlite XAD-2 column to give the pure product **20a** in an 88% yield. The compound **20b** was also obtained in a similar manner from 9-fluorenylmethoxycarbonyl-β-alanine (Fmoc-β-alanine) in an 86% yield.

20a: yellow solid; decomp. 230 ^oC; ^{1}H NMR (500 MHz, D_2O): δ 8.43 (1H, s), 7.62 (1H, d, *J* = 7.0 Hz), 7.57 (1H, d, *J* = 7.0 Hz), 7.04 (1H, d, *J* = 7.0 Hz), 6.84 (1H, t, *J* = 7.0 Hz), 6.55 (2H, m), 3.92 (3H, s), 3.68 (2H, s), 3.46 (2H, d, *J* = 5.0 Hz), 3.43 (2H, d, *J* = 5.0 Hz), 1.95 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ 168.0, 165.0, 148.0, 147,7, 143.9, 143.7, 138.0, 134.1, 130.3, 130.1, 125.4, 125.3, 122.7, 122.6, 122.4, 122.3, 113.0, 47.8, 41.7, 41.1, 40.3, 15.7; MS (FAB): *m/z* 390 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{22}H_{24}N_5O_2^+$: 390.1930, found: 390.1935.

20b: yellow solid; decomp. 335 °C; ^{1}H NMR (500 MHz, D_2O): δ9.99 (1H, s), 8.46 (2H, m), 8.37 (1H, m), 7.76 (1H, t, *J* = 7.0 Hz), 7.69 (1H, t, *J* = 7.0 Hz), 7.47 (1H, m), 4.51 (3H, s), 3.74 (2H, t, *J* = 6.5 Hz), 3.63 (2H, t, *J* = 6.5 Hz), 3.41 (3H, s), 3.19 (2H, t, *J* = 6.5 Hz), 2.66 (2H, t, *J* = 6.5 Hz); ^{13}C NMR (125 MHz, CD_3OD): δ172.8, 167.6, 148.0, 144.1, 143.8, 138.2, 134.0, 133.1, 130.3, 128.6, 125.4, 122.9, 122.7, 122.3, 121.3, 113.0, 111.6, 47.8, 41.2, 40.3, 37.2, 33.3, 15.7; MS (FAB): *m/z* 404 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{23}H_{26}N_5O_2^+$: 404.2087, found: 404.2090.

4.2.11. Synthesis of urea conjugates of pyridocarbazole derivatives with a peptide linker, ***21a,b***

To a cold solution of **20a** (20 mg, 0.047 mmol) and DIPEA (19 mg, 0.14 mmol) in DMF (1 mL) was slowly added 4-nitrophenyl *N*-methyl-*N*-nitrosocarbamate (**6a**) (11 mg, 0.056 mmol) in DMF (2 mL) at 0 °C under an argon atmosphere. The reaction mixture was stirred at the same temperature for 7 h. After the volatile materials were removed from the reaction mixture under reduced pressure, the residue was purified by ODS chromatography (a mixture of phosphate buffer (pH 3.3) and MeOH), and successively desalted via an Amberlite XAD-2 column to obtain the pure **21a** in an 88 % yield. The compounds **21b** was obtained in a similar manner from **20b** in a 73 % yield.

21a: yellow solid; decomp. 280 °C; ^{1}H NMR (500 MHz, D_2O): δ8.02 (1H, s), 7.63 (1H, d, *J* = 7.0 Hz), 7.25 (1H, d, *J* = 7.0 Hz), 6.99 (1H, d, *J* = 7.0 Hz), 6.95 (1H, d, *J* = 7.0 Hz), 6.78 (1H, d, 7.0 Hz), 6.59 (1H, t, *J* = 7.0 Hz), 3.67 (5H, m), 3.42 (4H, m), 2.43 (3H, s), 1.77 (3H, s); ^{13}C NMR (125 MHz, CD_3OD): δ174.5, 167.5, 161.6, 147.8, 144.0, 143.6, 138.0, 134.0, 133.1, 130.2, 128.5, 125.3, 122.8, 122.6, 122.4, 121.2, 113.1, 111.4, 47.8, 44.8, 41.4, 40.4, 27.0, 15.7; MS (FAB): *m/z* 447 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{24}H_{27}N_6O_3^+$: 447.2145, found 447.2138.

21b: yellow solid; decomp. 210 °C; ^{1}H NMR (500 MHz, D_2O): δ8.68 (1H, s), 7.14 (2H, m), 7.32 (1H, d, *J* = 7.0 Hz), 7.04 (1H, t, *J* = 7.0 Hz), 6.81 (1H, d, *J* = 7.0 Hz), 6.78 (1H, d, *J* = 7.0 Hz), 4.04 (3H, s), 3.52 (2H, t, *J* = 5.0 Hz), 3.45 (2H, t, *J* = 5.0 Hz), 3.17 (2H, t, *J* = 6.5 Hz), 2.31 (2H, t, *J* = 6.5 Hz), 2.22 (6H, m); ^{13}C NMR (125 MHz, CD_3OD): δ174.9, 169.0, 161.6, 149.5, 145.6, 136.7, 133.6, 132.7, 132.6, 130.4, 129.6, 125.1, 124.1, 121.4, 121.3, 120.2, 120.1, 114.9, 46.9, 41.0, 40.3, 38.2, 37.8, 26.8, 15.2; MS (FAB): *m/z* 461 (M^+ - Cl^-); HRMS (FAB): Calcd for $C_{25}H_{29}N_6O_3^+$: 461.2301, found 461.2295.

4.2.12. Synthesis of 2,2'-oxybis(ethylamine) ***(9a)***

A mixture of bis(2-chloroethyl) ether (**13**) (300 mg, 2.1 mmol) and potassium phthalimide (1.17 g, 6.29 mmol) in DMF (5 mL) was stirred at 60 °C for 20 h. The reaction mixture was treated with water and extracted with $CHCl_3$ and dried over $MgSO_4$, and then concentrated. The crude product was purified by silica gel column chromatography ($CHCl_3$) to yield the corresponding bis(phthalimidylethyl) ether **15** in a 69% yield; mp 162.1-163.2 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ7.65 (8H, m), 3.78 (4H, t, *J* = 5.5 Hz), 3.66 (4H, t, *J* = 5.5 Hz); ^{13}C NMR (500 MHz, $CDCl_3$): δ 168.1, 133.7, 132.1, 123.1, 67.5, 37.2; MS (FAB) *m/z*: 365 (M+H).

A mixture of **15** (575 mg, 0.83 mmol) and 80% hydrazine hydrate (8.3 mmol) was stirred under reflux for 4 h in $CHCl_3$ (10 mL). The reaction mixture was treated with water and extracted with $CHCl_3$, and then dried ($MgSO_4$). After evaporation of the solvent, distillation gave the product **9a** in a 93% yield; bp 75-79 ^oC / 5 mmHg (lit.[32] bp 55-60 ^oC / 2 mmHg); ^1H NMR (500 MHz, DMSO-d_6): δ 8.31 (4H, br-s), 3.32 (4H, t, J = 5.5 Hz), 2.63 (4H, t, J = 5.5 Hz); ^{13}C NMR (500 MHz, DMSO-d_6): δ 72.7, 41.3; MS (FAB) *m/z*: 105 (M+H).

4.3. Cytotoxic activity

4.3.1. Cell lines and culture

Sarcoma-180 and HeLa S-3 cells were provided by Dr. Okada (Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Japan), NIH3T3 cells were purchased from Sumitomo Dainippon Pharma Co., Ltd., and L1210 cells were provided by Dr. Ikekita (Department of Applied Biological Science, Tokyo University of Science, Japan). The cells were maintained in MEM (Nissui) supplemented with 10% FBS (GIBCO), 2% HEPES, 3% NEAA, and 2 mM L-glutamine in a water-saturated atmosphere of 5% CO_2 at 37 ^oC.

4.3.2. Cell viability assay

Cell viability was determined using a 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) assay, which is a method for determining cell viability by measuring the mitochondrial dehydrogenase action. Cells were seeded in a 96-well cell culture cluster (Becton Dickinson) at a density of 2×10^4 cells/mL and cultured 3 h prior to drug treatment. Cells were exposed at 37 ^oC for 24 - 72 h to pyridocarbazole derivatives. An MTT reagent was prepared at a concentration of 2 mg/mL in Dulbecco**'**s PBS without calcium and magnesium, and stored at 4 ^oC. After treatment for indicated times, cells were incubated with MTT reagent for 4 h at 37 ^oC. The plate was centrifuged at 3,000 rpm for 10 min, and the medium was removed. To solubilize the resultant MTT formazan, 200 μL/well of dimethyl sulfoxide (DMSO) was added to each well followed by thorough mixing with a mechanical plate mixer. Absorbance at 540 nm was measured on a microplate reader (MTP-500, CORONA), and the percentage of cell viability was taken as the percentage of absorbance at 540 nm of pyridocarbazole-treated cells against control cells.

4.4. Pharmacology

*4.4.1. Hoechst 33342 staining and microscopic observation of cells treated with pyridocarbazolium chloride **5aa**, and pyridocarbazole **17aa***

Sarcoma-180 cells (2.0×10^4 cells/plate) were treated with the vehicle alone (0.1% DMSO) for 24 h at 37 ^oC and stained with Hoechst 33342 (1 μg/ml) for 40 min at 37 ^oC as a control. The cells were also treated with 0.12 μM of either **5aa** or **17aa** for 24 h at 37 ^oC. The treated-cells were observed for evaluation of cellular uptake of these compounds under a fluorescence microscopic system.

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Supplementary Material (see footnote on the first page of this article): Copies of the ^{1}H NMR, ^{13}C NMR, MS, and IR spectra for key intermediates and final products.