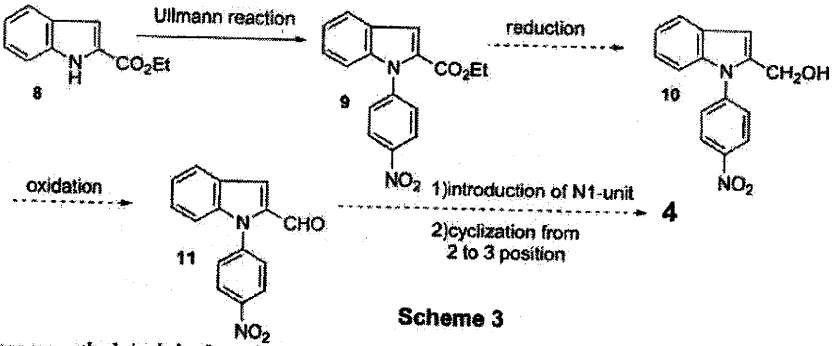
RESULTS AND DISCUSSION

The synthetic strategy for the synthesis of the relay compound **4** was designed as shown in Scheme 3. The key point is the initial (4-nitro)phenylation at the 1-nitrogen position in the indole nucleus, followed by elongation of the 2-substituent and cyclization to form the pyridine nucleus.

The usual construction of the pyridine ring in the indole nucleus for synthesis of the 9*H*-pyrido[3,4-*b*]indole nucleus is cyclization of the 3-substituent of the tryptamine derivative to the 2-position of the indole nucleus as seen in the Bischler-Napieralski reaction, Pictet-Spengler reaction and so on. On the other hand, there are few methods for cyclization of the 2-substituent to the 3-position of the indole nucleus. Several years ago we developed a method for 9*H*-pyrido[3,4-*b*]indole synthesis of the latter type in the course of the synthetic study of 4-oxo-β-carboline.[5] We applied this method in the present strategy.

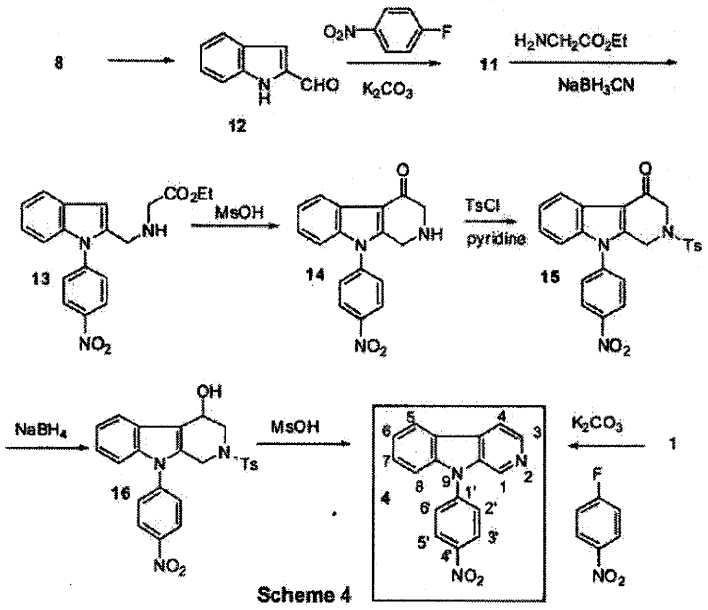


Scheme 3

For this purpose, ethyl indole-2-carboxylate **8** was allowed to react with 4-fluoronitrobenzene to give ethyl *N*-(4'-nitrophenyl)indole-2-carboxylate **9**. However, the reduction of ester carbonyl of **9** with $LiAlH_4$ was not successful (Scheme 3). Thus, the reaction scheme to prepare the aldehyde **11** had to be changed. The synthetic route was changed as in Scheme 4.

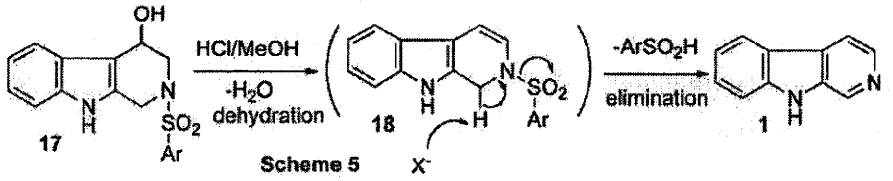
The first *N*-(4-nitro)phenylation of indole-2-carboxaldehyde[5] **12** prepared from **8**, which we feared to proceed with difficulty due to the sensitive reactivity of aldehyde functionality, went much better than expected (51% yield). The *N*-(4'-nitrophenyl)indole-2-carboxaldehyde **11** thus obtained was allowed to react with ethyl aminoacetate and then sodium cyanoborohydride to give the *N*-indolic aminoacetate **13**. The cyclization of **13** with methanesulfonic acid gave the cyclized amino ketone **14**. The aminoketone **14** was treated with tosyl chloride in the presence of pyridine to give the corresponding tosylamide **15** in good yield. The subsequent process of cyclic amino ketone resembling **14** to the target 9*H*-pyrido[3,4-*b*]indole nucleus has already been developed.[6]

The reduction of the ketone of **15** to the hydroxyl group with a large excess amount of sodium borohydride proceeded to give the alcohol **16** in good yield. The last and important dehydration and aromatization processes (two successive β-eliminations) were examined for the present reaction.



Scheme 4

The reaction[6] involved dehydration of the alcohol and β-elimination process around the sulfonyl group with HCl in MeOH, as shown in Scheme 5. In the present case the reaction did not proceed well with HCl in MeOH in several trials, probably due to its insolubility.



Scheme 5

After several acidic conditions were tried, the alcohol **16** was allowed to react with methanesulfonic acid. The target compound **4** was finally obtained from the basic layer in this reaction (20% yield). The product was identified with the already[2] and freshly synthesized sample directly via the Ullmann reaction from **1** and 4-bromo-(or 4-fluoro)nitrobenzene in the presence of K_2CO_3. It was proved that the Ullmann reaction of **1** proceeded at the indolic NH position even on the 9*H*-pyrido[3,4-*b*]indole nucleus. It is worth noting that the Ullmann reaction of **1** with 4-fluoronitrobenzene without K_2CO_3 did not proceed at all. This means that the formation of nitrogen anion is necessary for Ullmann reaction of indole and pyridine nitrogen cannot take Ullmann reaction directly. Thus, the structure **2** was chemically determined. Using this scheme, it may be possible to develop a new strategy for 9*H*-pyrido[3,4-*b*]indole synthesis that involves cyclication of the 2-substituent toward the 3-position of the indole skeleton.

EXPERIMENTAL

All melting points were measured on a hot stage micro-melting points apparatus (Yanagimoto) and are uncorrected. Elemental analyses were conducted with a Yanaco CHN CORDER MT-6. The ^{1}H-NMR spectra were measured with a Bruker Ultrashield™ 400 Plus (400MHz) spectrometer. Deuteriochloroform was used as the solvent with tetramethylsilane as an internal reference. MS spectra were measured on JEOL JMS-GC-mate II and JEOL JMS-600H spectrometers. IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer. For column chromatography, Silica gel 60 (70-230 mesh ASTM; Merck) was used.

1-(4'-Nitrophenyl)indole-2-carboxaldehyde 11

A solution of indole-2-carboxaldehyde[5] **12** (1.80 g, 12.4 mmol), 4-fluoronitrobenzene (5.19 g, 36.8 mmol), and powdered anhydrous K_2CO_3 (5.14 g, 37.2 mmol) in anhydrous DMF (27 mL) was heated with stirring at 100 °C for 1.5 h. The reaction mixture was poured onto water (150 mL), and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The residue (6.38 g) was chromatographed over SiO_2 with toluene as eluent to give the target compound **11** (1.70 g, 51%). Recrystallization of a part of the compound from a mixture of AcOEt and hexane gave pale yellow columns, mp 170-172 °C. *Anal.* Calcd for $C_{15}H_{10}N_2O_3$: C, 67.67; H, 3.79; N, 10.52. Found: C, 67.98; H, 3.95; N, 10.43. MS ($C_{15}H_{10}N_2O_3$): *m/z*266 (M^+). IR ν max(KBr)cm^{-1}: 1683 (sh), 1672 (CO). ^{1}H-NMR ($CDCl_3$) δ : 7.23-7.58 (6H, m, $C_{3,5,6,7,2',6'}$-H), 7.83 (2H, m, C_4-H), 8.41 (2H, d, *J*=8.0 Hz, $C_{3'}$,$C_{5'}$-H), 9.89 (1H, s, CHO).

Ethyl [1-(4'-Nitrophenyl)indole-2-ylmethyl]aminoacetate 13

To a muddy solution of 1-(4'-nitrophenyl)indole-2-carboxaldehyde **11** (724 mg, 2.77 mmol) and ethyl aminoacetate hydrochloride (1.12 g, 8.16 mmol) in ethanol (30 mL) was added triethylamine (1.17 mL, 8.16 mmol) and $NaBH_3CN$ (685 mg, 10.9 mmol) successively with stirring under ice-cooling. The reaction mixture (muddy state) was stirred under ice-cooling for 15 min and then at rt for an additional 3 h. Then, the reaction mixture was poured onto water and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to dryness to give a pale yellow residue. The crude products were chromatographed over SiO_2. Elution with toluene, followed by toluene-AcOEt (10:1), gave the target compound **13** (713 mg, 74%) as a pale yellow oil. MS ($C_{19}H_{19}N_3O_4$): *m/z* 353 (M^+). HRMS: Calcd for $C_{19}H_{19}N_3O_4$, 353.1376; Found, 353.1378. IR ν max($CHCl_3$)cm^{-1}: 3684, 3620 (NH), 1734 (C=O). ^{1}H-NMR δ :1.23 (3H, t, *J*=8.0 Hz, -$CH_2C\underline{H}_3$), 3.41 (2H, s, -CH_2NH-), 3.94 (2H, s, -NCH_2CO-), 4.12 (2H, *J*=8.0 Hz, -$OC\underline{H}_2CH_3$), 6.75 (1H, s, C_3-H), 7.18-7.26 (3H, m, $C_{5,6,7}$-H), 7.63 (1H, m, C_4-H), 7.73 (2H, *J*=8.0 Hz, $C_{2',6'}$-H), 8.41 (2H, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-one 14

A mixture of ethyl [1-(4'-nitrophenyl)indole-2-ylmethyl]aminoacetate **13** (513 mg, 1.45 mmol) and methanesulfonic acid (7 mL) was stirred at 45 °C for 45 min, and then 70 °C for 1 h. The reaction mixture was poured onto water (50 mL), basified with K_2CO_3, and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave a solid (425 mg). This solid was chromatographed over SiO_2 (12 g). Elution with $CHCl_3$, followed by AcOEt, gave a small amount of the starting material and unknown compounds. Further elution with a mixture of AcOEt and EtOH (10:1) gave the target compound **14** (298 mg, 67%). A part of the sample was recrystallized from acetone to give pale yellow fine needles, mp 215-217 °C (decomp). MS ($C_{17}H_{13}N_3O_3$): *m/z*307 (M^+, 25% of base peak), 252(base peak). HRMS: Calcd for $C_{17}H_{13}N_3O_3$, 307.0957; Found,307.0964. IR ν max(KBr)cm^{-1}:3326(NH), 1649(CO). ^{1}H-NMR δ : 2.23 (1H, br.s, NH), 3.67 (2H, s, C_3-H), 4.13 (2H, s, C_1-H), 7.21-7.45 (3H, m, $C_{6,7,8}$-H), 7.62 (2H, d, *J*=8.0 Hz, $C_{2',6'}$-H), 8.30 (1H. m, C_5-H), 8.50 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-2-tosyl-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-one 15

To a suspension of 9-(4'-nitrophenyl)-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole **14** (265 mg, 0.862 mmol) in pyridine (7 mL) was added TsCl (493 mg, 2.59 mmol) under ice-cooling. The mixture was stirred under ice-cooling for 15 min and at rt for an additional 1 h. The reaction mixture was poured onto water, extracted with $CHCl_3$, washed with dil. HCl aq. and water, and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the target compound **15** (349 mg, 88%). A part of the compound was recrystallized from a mixture of DMF and EtOH to give almost colorless very fine needles, mp 253-258 °C (decomp). *Anal.* Calcd for $C_{24}H_{19}N_3O_5S$: C, 62.46; H, 4.15; N, 9.11: Found: C; 62.42, H; 4.18, N; 8.58. MS: *m/z* 461 (M^+,15% of base peak), 306 (base peak). HRMS:Calcd for $C_{24}H_{19}N_3O_5S$, 461.1045; Found,461.1043. IR ν max(KBr)cm^{-1}:1664(CO). ^{1}H-NMR δ : 2.50 (3H, s, arom-CH_3), 4.11 (2H, s, C_3-H), 4.76 (2H, s, C_1-H), 7.20-7.45 (7H, m, arom-H), 7.87 (1H, dd, *J*=8.0 and 2.0 Hz, C_5-H), 7.92 (2H, d, *J*=8.0 Hz, Ts-ortho-H), 8.56 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-2-tosyl-2,3,4,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-ol 16

The tosyl ketone **15** (33 mg, 0.0715 mmol) was added to a mixture of $CHCl_3$ (1.5 mL) and MeOH (4 mL). To the resulting suspension was added $NaBH_4$ (270 mg, 7.15 mmol) under ice-cooling to prevent generation of heat at the beginning and then the whole was stirred for 4.5 h at rt. The reaction mixture was poured onto water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the target alcohol **16** as yellowish powder (31 mg, 94%). This sample showed one spot on TLC (SiO_2, toluene-AcOEt = 2:1), and was

used for the next reaction. A part of the powder was recrystallized from acetone-MeOH to give pale yellow powder, mp 175-177 °C (decomp). *Anal.* Calcd for $C_{24}H_{21}N_3O_5S$: C, 62.19; H, 4.57; N, 9.07. Found: C, 61.83; H, 4.68; N, 8.85. MS: 463 (M^+, 5.9% of the base peak), 252 (base peak). HRMS; Calcd, 463.1202; Found, 463.1208. IR ν max(KBr)cm^{-1}: 3482(OH). ^{1}H-NMR δ : 2.44 (3H, s, arom-CH_3), 3.16,3.96,4.55 (4H, aliph-H), 5.12 (1H, br. d, J=12.0 Hz, C_4-H), 7.24-7.78 (10H, m, arom-H), 8.46 (2H, d, J=8.0 Hz, $C_{3'}$, $C_{5'}$-H).

9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole 4 from 9-(4'-nitrophenyl)-2-(toluene-4"-sulfonyl)-2,3,4,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-ol 16

The above-mentioned alcohol **16** (40 mg, 0.086 mmol) was dissolved in methanesulfonic acid (3 mL) and stirred for 4 h at rt. The reaction mixture was poured onto water and extracted out with AcOEt. The aqueous layer was basified with K_2CO_3 and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the crude product. The crude product was purified with column-chromatography [SiO_2 (8 g), $CHCl_3$] to give yellow powder (5 mg, 20%), mp 188-190 °C. This sample was identified with the relay compound[2] derived from Ullmann reaction of norharman **1** and 4-bromonitrobenzene (or 4-fluoronitrobenzene) as described below, based on their NMR spectra and TLC behavior. ^{1}H-NMR δ : 7.44 (1H, m, C_6-H), 7.56-7.62 (2H, m, $C_{7,8}$-H), 7.85 (2H, d, J=8 Hz, $C_{2',6'}$-H), 8.05 (1H, d, J=4.0 Hz, C_4-H), 8.23 (1H,d, J=8.0 Hz, C_5-H), 8.53 (2H, d, J=8.0 Hz, $C_{3',5'}$-H), 8.59 (1H, br. d, J=4.0 Hz, C_3=H), 8.95(1H, s, C_1-H).

9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole 4 *via* Ullmann reaction[2] from norharman 1

A mixture of norharman **1** (40 mg, 0.238 mmol), 4-fluoronitrobenzene (66 mg, 0.476 mmol) and powdered anhydrous K_2CO_3 (99 mg, 714 mmol) was added to DMF (3 mL) and the whole was heated at 100 °C with stirring for 3 h. The reaction mixture was poured onto water (60 mL) and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The resulting mass was purified over column chromatography (SiO_2, $CHCl_3$) to give the target compound (79 mg, quantitative). This sample was recrystallized from $CHCl_3$-MeOH and then treated with $CHCl_3$ to give pale yellow needles, mp 192-192.5 °C. The sample obtained from recrystallization from $CHCl_3$-MeOH contained MeOH in its crystals. The crystals were dried at 100 °C *in vacuo* over night for elemental analysis. *Anal.* Calcd for $C_{17}H_{11}N_3O_2$: C; 70.58, H; 3.83, N; 14.53. Found: C; 70.60, H; 3.97, N; 14.53.

Ethyl 1-(4'-nitrophenyl)indole-2-carboxylate 9

In anhydrous DMF (3 mL) was added ethyl indole-2-carboxylate (299 mg, 1.58 mmol), 4-

fluoronitrobenzene (417 mg, 3 mmol), and powdered anhydrous K_2CO_3 (304 mg, 2.2 mmol). The whole was heated at 100 °C under stirring for 14.5 h. The reaction mixture was poured onto water, and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (702 mg) was chromatographed over SiO_2 and eluted with toluene to give the target compound **9** (266 mg, 54%). A part of this compound was recrystallized from AcOEt-hexane to give pale yellow plates, mp 133-135 °C. *Anal.* Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03. Found: C, 65.99; H, 4.64; N, 8.76. MS ($C_{17}H_{14}N_2O_4$): 310 (M^+). IR ν max(KBr)cm^{-1}: no NH, 1704 (CO). ^{1}H-NMR δ : 1.29 (3H, t, *J*=8.0 Hz, -$CH_2\underline{CH_3}$), 4.25 (2H, t, *J*=8.0 Hz, -$O\underline{CH_2}CH_3$), 7.13 (1H, d, *J*=1.5 Hz, C_3-H), 7.23-7.36 (3H, m, indolic Hs), 7.52-7.56 (3H, m, $C_{2'}$,$C_{6'}$,an indolic H), 7.76 (1H, d, *J*=9.0 Hz, C_4-H), 8.40 (2H, d, *J*=9.0 Hz, $C_{3'}$,$C_{5'}$-H).

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CHEMICAL CONFIRMATION OF THE STRUCTURE OF A MUTAGENIC AMINOPHENYLNORHARMAN, 9-(4'-AMINOPHENYL)-9*H*-PYRIDO[3,4-*b*]INDOLE : AN AUTHENTIC SYNTHESIS OF 9-(4'-NITROPHENYL)-9*H*- PYRIDO[3,4-*b*]INDOLE AS ITS RELAY COMPOUND

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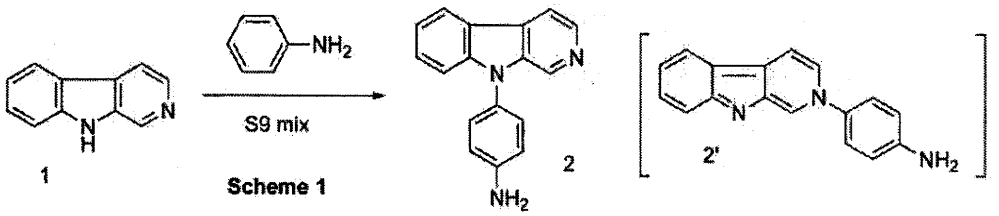
Abstract – 9-(4'-Aminophenyl)-9*H*-pyrido[3,4-*b*]indole **2** is a mutagenic compound produced by non-mutagenic norharman **1** and aniline in the presence of S9 mix. 9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole **4**, the relay compound for synthesis of **2**, was synthesized starting from ethyl indole-2-aldehyde **12** via initial *N*-(4-nitro)phenylation of the indole nucleus, elongation of the 2-aldehyde substituent, and then construction of the pyridine nucleus in order to ensure the nitrogen substitution in **2**.

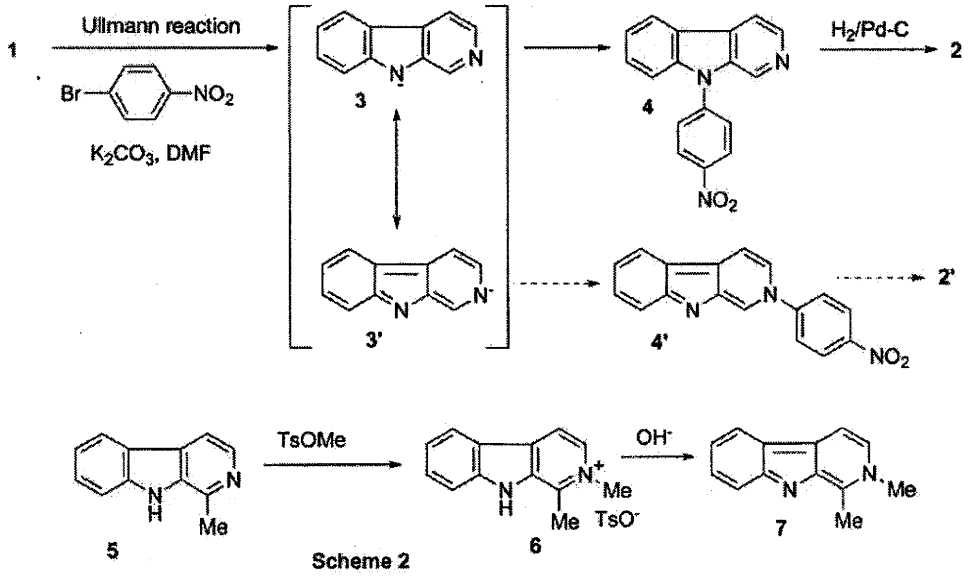
INTRODUCTION

Sugimura et al.[1] reported that norharman 1 (9*H*-pyrido[3,4-*b*]indole, β-carboline) itself is not mutagenic to *Salmonella* strains, but becomes mutagenic to *S. typhimurium* TA98 and YG1024 with S9 mix in the presence of non-mutagenic aromatic amines such as aniline and *o*-toluidine. In a



Scheme 1

subsequent report[2] they isolated mutagenic compound **2** produced by the reaction between norharman and aniline with S9 mix (Scheme 1). In order to elucidate the structure, one of the potential structures, 9-(4'-aminophenyl)-9*H*-pyrido[3,4-b]indole **2**, was synthesized[2] via Ullmann reaction of norharman **1** with 4-bromonitrobenzene, followed by catalytic hydrogenation. The synthetic sample was identical to the natural one and the spectral data of the product supported the structure of **2** but not **2'**. The synthetic strategy was based on the fact that Ullmann reaction of indoles with aryl halides proceeded at its NH position.[3] However, if the reaction occurs on the pyridine nitrogen of **1** via its basicity or another resonance structure **3'**, the product should be compound **4'** (Scheme 1 and 2), whose structure would be much more unstable than the structure **4**, as it has neither benzene, indole, nor pyridine aromaticity any longer. Thus, such a compound is thought to be difficult to produce. On the other hand, it was recently reported[4] that N_A-methylammonium harman **6** derived from harman **5** was basified to yield the compound **7**, whose skeleton is the same as those of **2'** and **4'** (Scheme 2). In this paper we report the authentic synthesis of the relay compound **4** in order to ensure the nitrogen substitution of the substituted phenyl group in **2**.

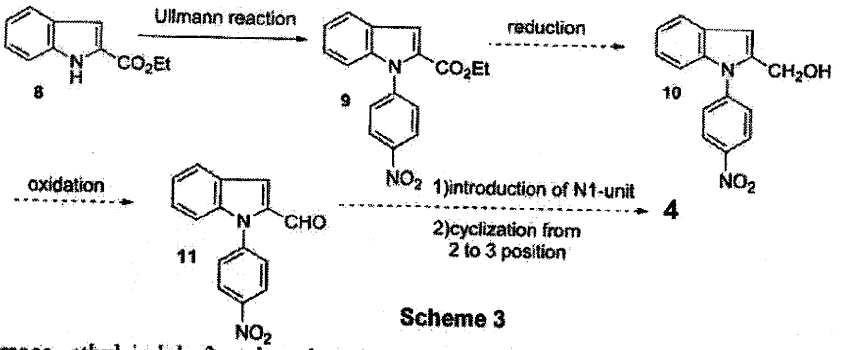


Scheme 2

RESULTS AND DISCUSSION

The synthetic strategy for the synthesis of the relay compound **4** was designed as shown in Scheme 3. The key point is the initial (4-nitro)phenylation at the 1-nitrogen position in the indole nucleus, followed by elongation of the 2-substituent and cyclization to form the pyridine nucleus.

The usual construction of the pyridine ring in the indole nucleus for synthesis of the 9*H*-pyrido[3,4-*b*]indole nucleus is cyclization of the 3-substituent of the tryptamine derivative to the 2-position of the indole nucleus as seen in the Bischler-Napieralski reaction, Pictet-Spengler reaction and so on. On the other hand, there are few methods for cyclization of the 2-substituent to the 3-position of the indole nucleus. Several years ago we developed a method for 9*H*-pyrido[3,4-*b*]indole synthesis of the latter type in the course of the synthetic study of 4-oxo-β-carboline.[5] We applied this method in the present strategy.

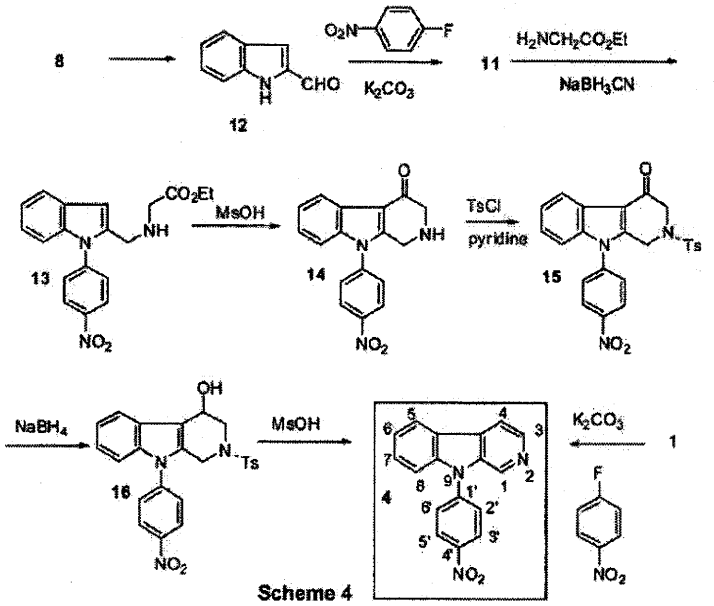


Scheme 3

For this purpose, ethyl indole-2-carboxylate **8** was allowed to react with 4-fluoronitrobenzene to give ethyl *N*-(4'-nitrophenyl)indole-2-carboxylate **9**. However, the reduction of ester carbonyl of **9** with $LiAlH_4$ was not successful (Scheme 3). Thus, the reaction scheme to prepare the aldehyde **11** had to be changed. The synthetic route was changed as in Scheme 4.

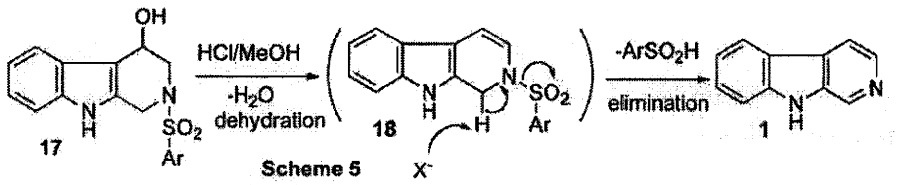
The first *N*-(4-nitro)phenylation of indole-2-carboxaldehyde[5] **12** prepared from **8**, which we feared to proceed with difficulty due to the sensitive reactivity of aldehyde functionality, went much better than expected (51% yield). The *N*-(4'-nitrophenyl)indole-2-carboxaldehyde **11** thus obtained was allowed to react with ethyl aminoacetate and then sodium cyanoborohydride to give the *N*-indolic aminoacetate **13**. The cyclization of **13** with methanesulfonic acid gave the cyclized amino ketone **14**. The aminoketone **14** was treated with tosyl chloride in the presence of pyridine to give the corresponding tosylamide **15** in good yield. The subsequent process of cyclic amino ketone resembling **14** to the target 9*H*-pyrido[3,4-*b*]indole nucleus has already been developed.[6]

The reduction of the ketone of **15** to the hydroxyl group with a large excess amount of sodium borohydride proceeded to give the alcohol **16** in good yield. The last and important dehydration and aromatization processes (two successive β-eliminations) were examined for the present reaction.



Scheme 4

The reaction[6] involved dehydration of the alcohol and β-elimination process around the sulfonyl group with HCl in MeOH, as shown in Scheme 5. In the present case the reaction did not proceed well with HCl in MeOH in several trials, probably due to its insolubility.



Scheme 5

After several acidic conditions were tried, the alcohol **16** was allowed to react with methanesulfonic acid. The target compound **4** was finally obtained from the basic layer in this reaction (20% yield). The product was identified with the already[2] and freshly synthesized sample directly via the Ullmann reaction from **1** and 4-bromo-(or 4-fluoro)nitrobenzene in the presence of K_2CO_3. It was proved that the Ullmann reaction of **1** proceeded at the indolic NH position even on the 9*H*-pyrido[3,4-*b*]indole nucleus. It is worth noting that the Ullmann reaction of 1 with 4-fluoronitrobenzene without K_2CO_3 did not proceed at all. This means that the formation of nitrogen anion is necessary for Ullmann reaction of indole and pyridine nitrogen cannot take Ullmann reaction directly. Thus, the structure **2** was chemically determined. Using this scheme, it may be possible to develop a new strategy for 9*H*-pyrido[3,4-*b*]indole synthesis that involves cyclication of the 2-substituent toward the 3-position of the indole skeleton.

EXPERIMENTAL

All melting points were measured on a hot stage micro-melting points apparatus (Yanagimoto) and are uncorrected. Elemental analyses were conducted with a Yanaco CHN CORDER MT-6. The ^{1}H-NMR spectra were measured with a Bruker Ultrashield™ 400 Plus (400MHz) spectrometer. Deuteriochloroform was used as the solvent with tetramethylsilane as an internal reference. MS spectra were measured on JEOL JMS-GC-mate II and JEOL JMS-600H spectrometers. IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer. For column chromatography, Silica gel 60 (70-230 mesh ASTM; Merck) was used.

1-(4'-Nitrophenyl)indole-2-carboxaldehyde 11

A solution of indole-2-carboxaldehyde[5] **12** (1.80 g, 12.4 mmol), 4-fluoronitrobenzene (5.19 g, 36.8 mmol), and powdered anhydrous K_2CO_3 (5.14 g, 37.2 mmol) in anhydrous DMF (27 mL) was heated with stirring at 100 °C for 1.5 h. The reaction mixture was poured onto water (150 mL), and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The residue (6.38 g) was chromatographed over SiO_2 with toluene as eluent to give the target compound **11** (1.70 g, 51%). Recrystallization of a part of the compound from a mixture of AcOEt and hexane gave pale yellow columns, mp 170-172 °C. *Anal.* Calcd for $C_{15}H_{10}N_2O_3$: C, 67.67; H, 3.79; N, 10.52. Found: C, 67.98; H, 3.95; N, 10.43. MS ($C_{15}H_{10}N_2O_3$): *m/z*266 (M^+). IR ν max(KBr)cm^{-1}: 1683 (sh), 1672 (CO). ^{1}H-NMR ($CDCl_3$) δ : 7.23-7.58 (6H, m, $C_{3,5,6,7,2',6'}$-H), 7.83 (2H, m, C_4-H), 8.41 (2H, d, *J*=8.0 Hz, $C_{3'}$,$C_{5'}$-H), 9.89 (1H, s, CHO).

Ethyl [1-(4'-Nitrophenyl)indole-2-ylmethyl]aminoacetate 13

To a muddy solution of 1-(4'-nitrophenyl)indole-2-carboxaldehyde **11** (724 mg, 2.77 mmol) and ethyl aminoacetate hydrochloride (1.12 g, 8.16 mmol) in ethanol (30 mL) was added triethylamine (1.17 mL, 8.16 mmol) and $NaBH_3CN$ (685 mg, 10.9 mmol) successively with stirring under ice-cooling. The reaction mixture (muddy state) was stirred under ice-cooling for 15 min and then at rt for an additional 3 h. Then, the reaction mixture was poured onto water and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to dryness to give a pale yellow residue. The crude products were chromatographed over SiO_2. Elution with toluene, followed by toluene-AcOEt (10:1), gave the target compound **13** (713 mg, 74%) as a pale yellow oil. MS ($C_{19}H_{19}N_3O_4$): *m/z* 353 (M^+). HRMS: Calcd for $C_{19}H_{19}N_3O_4$, 353.1376; Found, 353.1378. IR ν max($CHCl_3$)cm^{-1}: 3684, 3620 (NH), 1734 (C=O). ^{1}H-NMR δ :1.23 (3H, t, *J*=8.0 Hz, -$CH_2\underline{CH_3}$), 3.41 (2H, s, -CH_2NH-), 3.94 (2H, s, -NCH_2CO-), 4.12 (2H, *J*=8.0 Hz, -$OC\underline{H_2}CH_3$), 6.75 (1H, s, C_3-H), 7.18-7.26 (3H, m, $C_{5,6,7}$-H), 7.63 (1H, m, C_4-H), 7.73 (2H, *J*=8.0 Hz, $C_{2',6'}$-H), 8.41 (2H, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-one 14

A mixture of ethyl [1-(4'-nitrophenyl)indole-2-ylmethyl]aminoacetate **13** (513 mg, 1.45 mmol) and methanesulfonic acid (7 mL) was stirred at 45 °C for 45 min, and then 70 °C for 1 h. The reaction mixture was poured onto water (50 mL), basified with K_2CO_3, and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave a solid (425 mg). This solid was chromatographed over SiO_2 (12 g). Elution with $CHCl_3$, followed by AcOEt, gave a small amount of the starting material and unknown compounds. Further elution with a mixture of AcOEt and EtOH (10:1) gave the target compound **14** (298 mg, 67%). A part of the sample was recrystallized from acetone to give pale yellow fine needles, mp 215-217 °C (decomp). MS ($C_{17}H_{13}N_3O_3$): *m/z*307 (M^+, 25% of base peak), 252(base peak). HRMS: Calcd for $C_{17}H_{13}N_3O_3$, 307.0957; Found.307.0964. IR ν max(KBr)cm^{-1}:3326(NH), 1649(CO). ^{1}H-NMR δ : 2.23 (1H, br.s, NH), 3.67 (2H, s, C_3-H), 4.13 (2H, s, C_1-H), 7.21-7.45 (3H, m, $C_{6,7,8}$-H), 7.62 (2H, d, *J*=8.0 Hz, $C_{2',6'}$-H), 8.30 (1H, m, C_5-H), 8.50 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-2-tosyl-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-one 15

To a suspension of 9-(4'-nitrophenyl)-1,2,3,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole **14** (265 mg, 0.862 mmol) in pyridine (7 mL) was added TsCl (493 mg, 2.59 mmol) under ice-cooling. The mixture was stirred under ice-cooling for 15 min and at rt for an additional 1 h. The reaction mixture was poured onto water, extracted with $CHCl_3$, washed with dil. HCl aq. and water, and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the target compound **15** (349 mg, 88%). A part of the compound was recrystallized from a mixture of DMF and EtOH to give almost colorless very fine needles, mp 253-258 °C (decomp). *Anal.* Calcd for $C_{24}H_{19}N_3O_5S$: C, 62.46; H, 4.15; N, 9.11: Found: C; 62.42, H; 4.18, N; 8.58. MS: *m/z* 461 (M^+,15% of base peak), 306 (base peak). HRMS:Calcd for $C_{24}H_{19}N_3O_5S$, 461.1045; Found,461.1043. IR ν max(KBr)cm^{-1}:1664(CO). ^{1}H-NMR δ : 2.50 (3H, s, arom-CH_3), 4.11 (2H, s, C_3-H), 4.76 (2H, s, C_1-H), 7.20-7.45 (7H, m, arom-H), 7.87 (1H, dd, *J*=8.0 and 2.0 Hz, C_5-H), 7.92 (2H, d, *J*=8.0 Hz, Ts-ortho-H), 8.56 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H).

9-(4'-Nitrophenyl)-2-tosyl-2,3,4,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-ol 16

The tosyl ketone **15** (33 mg, 0.0715 mmol) was added to a mixture of $CHCl_3$ (1.5 mL) and MeOH (4 mL). To the resulting suspension was added $NaBH_4$ (270 mg, 7.15 mmol) under ice-cooling to prevent generation of heat at the beginning and then the whole was stirred for 4.5 h at rt. The reaction mixture was poured onto water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the target alcohol **16** as yellowish powder (31 mg, 94%). This sample showed one spot on TLC (SiO_2, toluene-AcOEt = 2:1), and was

used for the next reaction. A part of the powder was recrystallized from acetone-MeOH to give pale yellow powder, mp 175-177 °C (decomp). *Anal.* Calcd for $C_{24}H_{21}N_3O_5S$: C, 62.19; H, 4.57; N, 9.07. Found: C, 61.83; H, 4.68; N, 8.85. MS: 463 (M^+, 5.9% of the base peak), 252 (base peak). HRMS; Calcd, 463.1202; Found, 463.1208. IR ν max(KBr)cm^{-1}: 3482(OH). ^{1}H-NMR δ : 2.44 (3H, s, arom-CH_3), 3.16,3.96,4.55 (4H, aliph-H), 5.12 (1H, br. d, *J*=12.0 Hz, C_4-H), 7.24-7.78 (10H, m, arom-H), 8.46 (2H, d, *J*=8.0 Hz, $C_{3'}$, $C_{5'}$-H).

9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole 4 from 9-(4'-nitrophenyl)-2-(toluene-4"-sulfonyl)-2,3,4,9-tetrahydro-9*H*-pyrido[3,4-*b*]indole-4-ol 16

The above-mentioned alcohol **16** (40 mg, 0.086 mmol) was dissolved in methanesulfonic acid (3 mL) and stirred for 4 h at rt. The reaction mixture was poured onto water and extracted out with AcOEt. The aqueous layer was basified with K_2CO_3 and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* to dryness gave the crude product. The crude product was purified with column-chromatography [SiO_2 (8 g), $CHCl_3$] to give yellow powder (5 mg, 20%), mp 188-190 °C. This sample was identified with the relay compound[2] derived from Ullmann reaction of norharman **1** and 4-bromonitrobenzene (or 4-fluoronitrobenzene) as described below, based on their NMR spectra and TLC behavior. ^{1}H-NMR δ : 7.44 (1H, m, C_6-H), 7.56-7.62 (2H, m, $C_{7,8}$-H), 7.85 (2H, d, *J*=8 Hz, $C_{2',6'}$-H), 8.05 (1H, d, *J*=4.0 Hz, C_4-H), 8.23 (1H,d, *J*=8.0 Hz, C_5-H), 8.53 (2H, d, *J*=8.0 Hz, $C_{3',5'}$-H), 8.59 (1H, br. d, *J*=4.0 Hz, C_3=H), 8.95(1H, s, C_1-H).

9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole 4 *via* Ullmann reaction[2] from norharman 1

A mixture of norharman **1** (40 mg, 0.238 mmol), 4-fluoronitrobenzene (66 mg, 0.476 mmol) and powdered anhydrous K_2CO_3 (99 mg, 714 mmol) was added to DMF (3 mL) and the whole was heated at 100 °C with stirring for 3 h. The reaction mixture was poured onto water (60 mL) and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The resulting mass was purified over column chromatography (SiO_2, $CHCl_3$) to give the target compound (79 mg, quantitative). This sample was recrystallized from $CHCl_3$-MeOH and then treated with $CHCl_3$ to give pale yellow needles, mp 192-192.5 °C. The sample obtained from recrystallization from $CHCl_3$-MeOH contained MeOH in its crystals. The crystals were dried at 100 °C *in vacuo* over night for elemental analysis. *Anal.* Calcd for $C_{17}H_{11}N_3O_2$: C; 70.58, H; 3.83, N; 14.53. Found: C; 70.60, H; 3.97, N; 14.53.

Ethyl 1-(4'-nitrophenyl)indole-2-carboxylate 9

In anhydrous DMF (3 mL) was added ethyl indole-2-carboxylate (299 mg, 1.58 mmol), 4-

fluoronitrobenzene (417 mg, 3 mmol), and powdered anhydrous K_2CO_3 (304 mg, 2.2 mmol). The whole was heated at 100 °C under stirring for 14.5 h. The reaction mixture was poured onto water, and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (702 mg) was chromatographed over SiO_2 and eluted with toluene to give the target compound **9** (266 mg, 54%). A part of this compound was recrystallized from AcOEt-hexane to give pale yellow plates, mp 133-135 °C. *Anal.* Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03. Found: C, 65.99; H, 4.64; N, 8.76. MS ($C_{17}H_{14}N_2O_4$): 310 (M^+). IR ν max(KBr)cm^{-1}: no NH, 1704 (CO). ^{1}H-NMR δ : 1.29 (3H, t, *J*=8.0 Hz, -$CH_2\underline{CH_3}$), 4.25 (2H, t, *J*=8.0 Hz, -$O\underline{CH_2}CH_3$), 7.13 (1H, d, *J*=1.5 Hz, C_3-H), 7.23-7.36 (3H, m, indolic Hs), 7.52-7.56 (3H, m, $C_{2'}$,$C_{6'}$,an indolic H), 7.76 (1H, d, *J*=9.0 Hz, C_4-H), 8.40 (2H, d, *J*=9.0 Hz, $C_{3'}$,$C_{5'}$-H).

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Benzo[a]pyrene exposed to solar-simulated light inhibits apoptosis and augments carcinogenicity

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) are widespread environmental pollutants and several lines of experimental evidence have suggested a role in carcinogenesis. PAHs in the environment are exposed to sunlight and photomodified PAHs have been detected in contaminated sediment and air particulate matter; however, the carcinogenicity of photomodified PAHs is not well understood. In this study, we found that solar-simulated light-irradiated BaP (LBaP) inhibited apoptosis, leading to cancer. LBaP suppressed apoptosis induced by cell detachment and serum depletion in a dose and light-irradiated time-dependent manner. The antiapoptotic effect was related to the production of reactive oxygen species from degraded BaP. The cells that survived apoptosis by LBaP treatment were transformed having the ability to form colonies in soft agar and tumors in nude mice. These capabilities were specific to LBaP, not BaP itself. The results suggested that the carcinogenicity of PAHs may be attributable not only to the genetic damage induced by their metabolites, but also to the antiapoptotic effects of oxidative products on exposure to sunlight.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) are widespread environmental pollutants formed as byproducts of combustion. Several lines of experimental evidence have suggested a role for PAHs in carcinogenesis [1]. PAHs themselves show no genotoxicity, but biological activation through oxidative metabolism by cytochrome P450-, peroxidase- and/or microsomal epoxide hydrolase-catalyzed reactions, can make them genotoxic. The ultimate carcinogenic metabolite of BaP is anti-7,8-dihydrodiol-9,10-epoxy-benzo[a]pyrene (BPDE) which has the ability to form stable adducts with DNA and can act as an initiator of carcinogenesis. A large proportion of the metabolic profile of BaP includes the BaP-quinones; however, the mechanism by which they may act as carcinogens is little understood. BaP-quinones are produced biologically by cytochrome P450 isoenzymes and peroxidases, as well as environmentally through sunlight [2,3].

Due to a multiple aromatic ring system, BaP can absorb sunlight in the visible (400–700 nm) and ultraviolet (UV) regions (290–400 nm) of the solar spectrum, causing structural modification (photomodification). Photomodified BaPs, BaP diones (BaP-1,6-dione, BaP-3,6-dione and BaP-6,12-dione) have been detected in particulate matter found in air [2], and hydroxy-BaPs like BaP-4,5-dihydrodiol have also been identified as photoproducts [4]. Photomodified compounds are generally produced via an oxidation and are in some cases more toxic than their parental compounds [5,6]. Oxidized BaPs like BaP-quinones are highly chemically active compounds that have been demonstrated in chemical systems to undergo one electron redox cycling with their semiquinone radicals resulting in the formation of reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals [7]. BaP-quinones such as BaP-1,6-dione, BaP-3,6-dione and BaP-6,12-dione show significant cytotoxicity and genotoxicity [6,8]. BaP-7,8-dione causes mutations in p53, one of the most commonly mutated tumor suppressor genes in cancer, through the production of ROS [9,10].

Burdick et al. [11] reported that BaP-quinones, through the generation of H_2O_2, increased the proliferation of breast tumor cells. ROS have been proposed to be involved in tumor progression and metastasis [12]. H_2O_2 increases the tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) [13,14]. The signaling enhances mitogen-activated protein kinases (MAPKs) and the phosphoinositide 3-kinase (PI3-kinase)/Akt pathway [14–16], resulting in cell proliferation and survival. These findings indicated that a certain dose of ROS enhances a pro-survival pathway, although excess ROS would damage cells and result in cell death. Production of ROS by photomodified PAHs may elicit similar pro-

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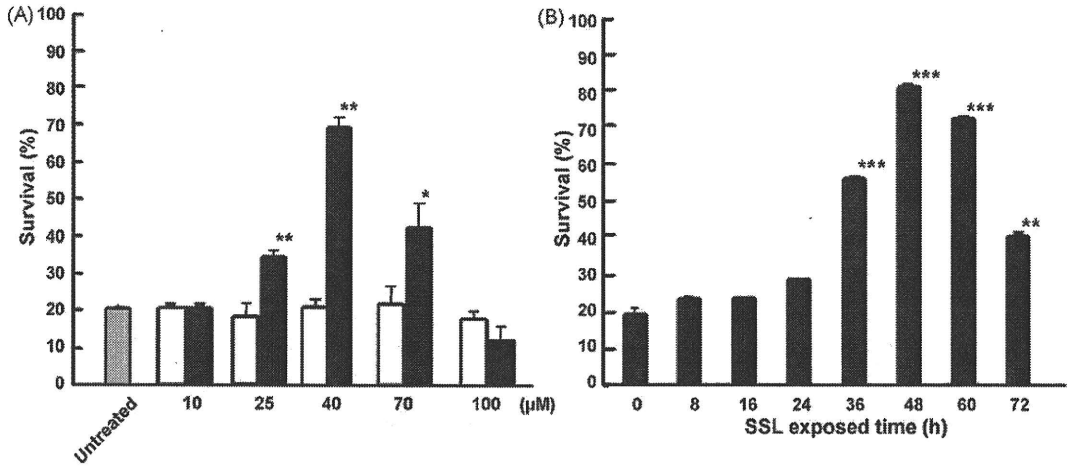

Fig. 1. Inhibition of cell death by LBaP. NIH3T3 cells trypsinized and plated in BSA-coated dishes were treated with BaP or LBaP for 2 h and washed twice with serum-free DMEM. After being cultured in serum-free DMEM for 24 h, they were loaded with FDA and survival was determined using FCM. (A) BaP (0.36 mM) was exposed to SSL for 48 h and added at several concentrations. Gray column: untreated, open column: BaP, closed column: LBaP. (B) BaP was exposed to SSL for 8–72 h and added at a concentration of 40 μM. Values are means ± S.D. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

survival signals. BaP-quinones phosphorylated EGFR and activated the downstream signaling as signal transducers and activators of transcription (STATs) [17]. BPDE has been shown to suppress apoptosis via activation of an Akt pathway in mammary epithelial cells [18]. We also have shown that exposure to benzene metabolites and ultraviolet rays suppressed the apoptosis induced by serum-starvation and loss of adhesion [19–21]. This was attributable to the production of ROS, especially H_2O_2, which activates a PI3-kinase/Akt pathway. The cells that avoided apoptosis were confirmed to be transformed, indicating that the inhibition of apoptosis (pro-survival signaling) has some roles in tumor initiation and promotion [20]. As oxidized BaP was thought to produce ROS and to activate pro-survival pathways like the PI3-kinase/Akt and MAPK pathways, we considered that sunlight-exposed BaP needed to be examined for both genotoxicity and apoptosis-inhibitory activity.

In the present study, we have found that photooxidized BaP inhibited apoptosis. The cells which escaped apoptosis were transformed, showing anchorage-independent growth and forming tumors in nude mice. This paper provides a mechanism for the carcinogenicity of PAHs in terms of photodegradation of PAHs and inhibition of apoptosis.

2. Materials and methods

2.1. Materials

Benzo[a]pyrene (BaP) and bis-benzimide (Hoechst33342) were obtained from Sigma–Aldrich Co. (St. Louis, MO). Bovine serum albumin (BSA) and fluorescein diacetate (FDA) were purchased from Wako Pure Chemicals Ind. Ltd. (Osaka, Japan). 6-Carboxy-2,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCFH-DA) was purchased from Molecular Probes Inc. (Eugene, OR). The caspase-3/7 substrate, acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA), was obtained from Peptide Inst. Inc. (Osaka, Japan).

2.2. Exposure of BaP to solar-simulated light

Light-irradiated BaP was prepared as described in our previous paper [22]. Briefly, 0.36 mM BaP was dissolved in a mixed solvent of methanol and acetone (methanol:acetone = 8:3) in quartz test tubes. The BaP was exposed to three solar-simulated light (SSL) tubes (TRUE-LITE: 18 W, 580 nm, Duro-Test Lighting, Inc., PA) with rotation at 25 °C. After a given period of irradiation, the solvent was evaporated and redissolved in dimethylsulfoxide (DMSO) at a final concentration of 13.2 mM. This preparation is herein referred to as "LBaP" (light-irradiated BaP).

2.3. Cell culture and cell treatment

NIH3T3 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) in a 37 °C humidified atmosphere containing 5% CO_2 in air. For the experiments, the cells were seeded at 3×10^6 cells/100 mm tissue culture dish. They were exponentially growing. After culture for 6 h, the medium was changed to serum-free DMEM. Following a further culture for 18 h, the cells were trypsinized and plated in cell suspension dishes previously coated with heat-denatured BSA (2 mg/ml) at 37 °C for 1 h (1×10^5 cells/well of 24-well plates or 5×10^5 cells/35 mm dish). The cells were treated with several concentrations of BaP or LBaP for 2 h and washed twice with serum-free DMEM. The concentrations of DMSO in medium were 0.038–0.758% according to the treated concentrations of BaP or LBaP (5–100 μM). The DMSO showed no effect on cell survival. In 'untreated', 0.3% of DMSO was treated.

2.4. Viability assay

BaP or LBaP-treated cells were further cultured for 24 h in the absence of CS, washed with phosphate-buffered saline (PBS), suspended in PBS containing FDA (0.1 μg/ml) and incubated for 10 min at 37 °C. Viability was determined using a flow cytometer (FCM) (Epics XL; Coulter, Hialeah, FL).

2.5. Determination of apoptotic cells

Apoptosis was measured by detecting both chromatin-condensed cells and DNA fragmentation as described previously [19]. Briefly, for the detection of chromatin-condensed cells, LBaP-treated cells were cultured for 22 h in the absence of CS and stained with the DNA-binding fluorochrome Hoechst33342. Following incubation for 10 min at room temperature, the cells were scored microscopically. For the detection of DNA fragmentation, LBaP-treated cells were cultured for 12 h in the absence of CS and resuspended in a 1% low-melting agarose solution (5×10^5 cells/90 μl), which was placed in an agarose plug maker. Solidi-

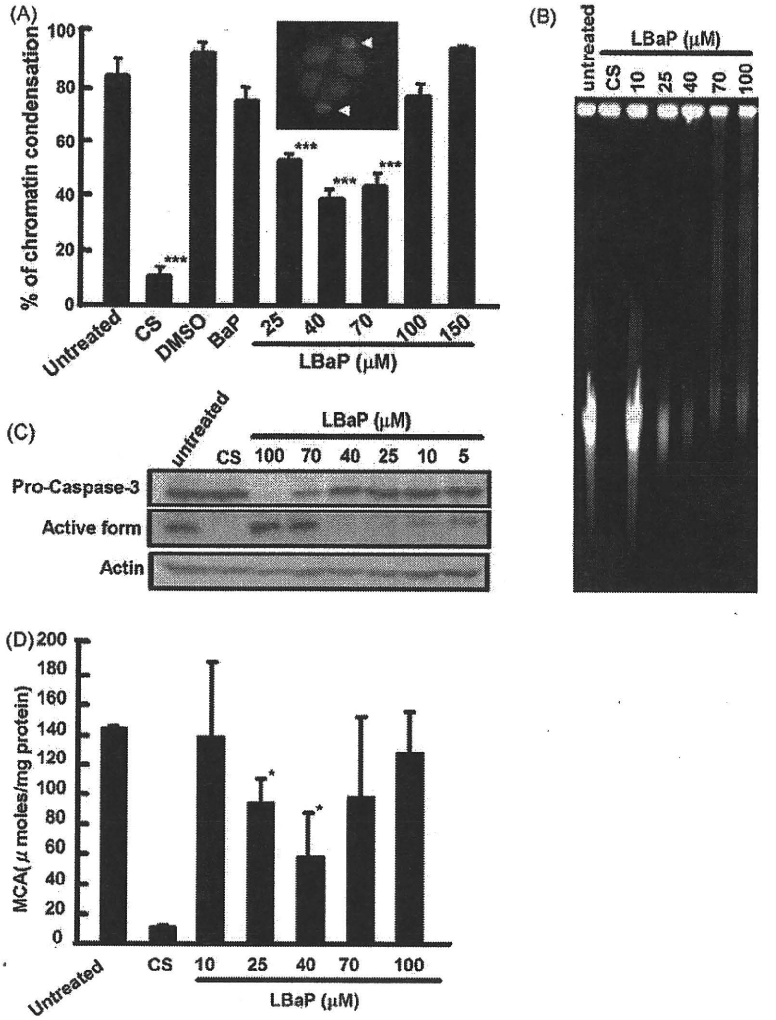

Fig. 2. Inhibition of apoptosis by LBaP. NIH3T3 cells trypsinized and plated in BSA-coated dishes were treated with several concentrations of LBaP for 2 h and washed twice with serum-free DMEM. (A) Chromatin condensation. The cells were further cultured for 22 h in serum-free DMEM and fixed in 2% glutaraldehyde for 2 h. They were stained with Hoechst33342 for 10 min and chromatin condensed cells were scored microscopically. Arrows in image: chromatin condensed cells. CS means the addition of CS during the experiment. (B) DNA fragmentation. The cells were further cultured for 12 h in serum-free DMEM and built in a 1% agarose solution. Solidified agarose plugs were placed in 0.8% agarose gel and BSFGE was performed in 0.5× TBE buffer. The DNA in the gel was visualized using ethidium bromide. (C) Degradation of procasapse-3. The cells were further cultured for 6 h in serum-free DMEM and lysed for Western blotting. (D) Caspase-3/7 activity. The cells were further cultured for 6 h in serum-free DMEM and lysed in lysis buffer on ice. An aliquot of cell lysate (25 μg proteins) was added to a reaction buffer containing 250 μM fluorogenic substrate (Ac-DEVD-MCA) and incubated for 1 h at 37 °C. The amounts of fluorogenic MCA moiety released were measured using a spectrofluorometer and converted to micromoles of MCA released using the standard curve of 7-amino-4-methylcoumarin. Values are means ± S.D. $^{*}p < 0.05$, $^{***}p < 0.001$.

fied agarose plugs incubated in proteinase k solution for 48 h were placed in a 0.8% agarose gel and electrophoresis was performed in 0.5× Tris–borate EDTA buffer with a biased sinusoidal field gel electrophoresis (BSFGE) system (Atto. Co., Japan). The DNA in the gel was visualized using ethidium bromide.

2.6. Determination of caspase-3/7 activity

Caspase-3/7 activity was measured by a direct assay of the caspase activity in cell lysates as described previously [19]. Briefly, following incubation for 6 h after treatment with LBaP for 2 h, the cells were lysed and aliquots were incubated in the presence of Ac-DEVD-MCA (250 μM) for 1 h at 37 °C. The amounts of fluorogenic MCA moiety released were measured using a spectrofluorometer (FL4500, Hitachi, Tokyo, Japan) (ex. 380 nm, em. 460 nm).

The degradation of procaspase-3 was examined by Western blotting. Following incubation for 6 h after treatment with LBaP for 2 h, the cells were lysed and a definite amount of protein was separated by 12.5% SDS–polyacrylamide gel electrophoresis (PAGE), then blotted onto polyvinylidene fluoride (PVDF) transfer membranes. After the blocking of nonspecific binding with 10% skim milk, the membranes were incubated with anti-caspase-3 polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) used at a dilution of 1:1000 overnight at 4 °C and washed with T-PBS (0.1% Tween 20 in PBS). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000). Signals were detected with the enhanced chemiluminescence detection system (GE Healthcare, UK).

2.7. Flow cytometric detection of intracellular peroxide

Immediately after treatment with LBaP for 1 h, the cells were incubated for 1 h in the presence of DCFH-DA (10 μM). They were suspended in PBS, and then analyzed with FCM.

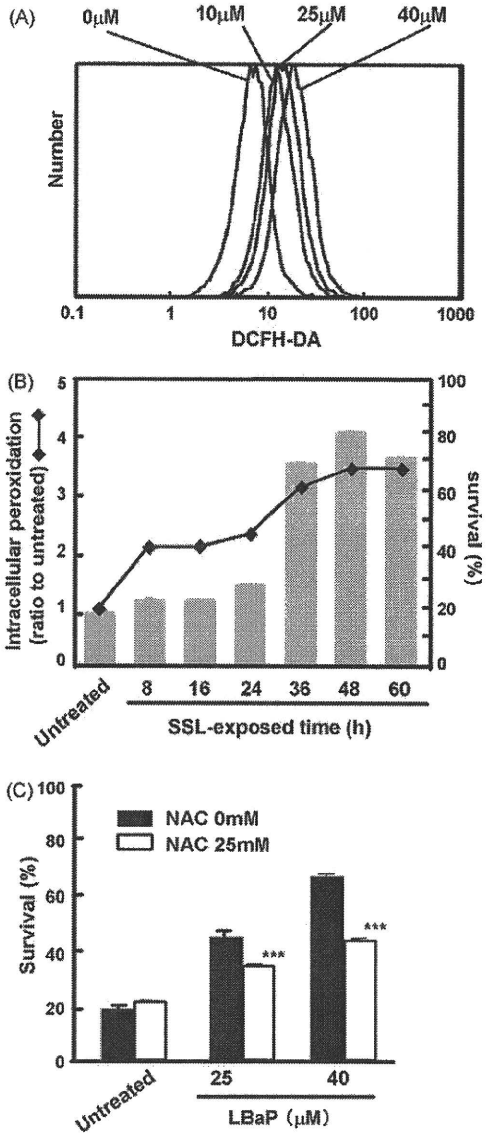

Fig. 3. The relationship between the antiapoptotic effect of LBaP and production of ROS. (A) Intracellular peroxidation after treatment with LBaP. NIH3T3 cells trypsinized and plated in BSA-coated dishes were treated with several concentrations of LBaP for 1 h and cultured for 1 h in the presence of DCFH-DA (10 μM). They were suspended in PBS, then analyzed with FCM. (B) Correlation of intracellular peroxidation with cell survival. Intracellular peroxidation was calculated from the top peaks of DCFH-DA in FCM histograms and presented relative to the untreated control. Survival after treatment with LBaP (40 μM) was determined as described in the legend to Fig. 1. (C) Survival after treatment with LBaP in the presence of NAC. NAC (25 mM) was added for 30 min before and during treatment with LBaP (25 and 40 μM). Survival was determined as described in the legend to Fig. 1.

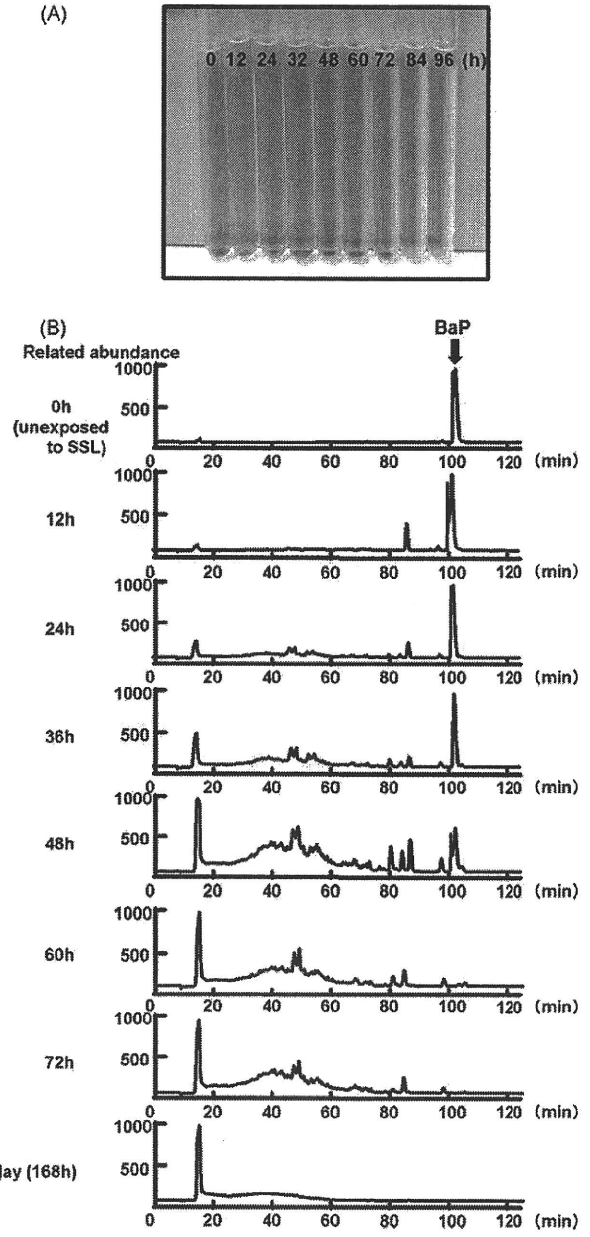

Fig. 4. Degradation of BaP on exposure to SSL. BaP (0.36 mM) was exposed to SSL for 12–168 h. (A) Change in appearance of BaP exposed to SSL. (B) HPLC chromatograms of BaP exposed to SSL. BaP (0 h) and LBaP concentrated at 52.8 mM were diluted tenfold with methanol and 100 μl was injected into the HPLC system.

2.8. Determination of colony-forming ability in soft agar

As an index of tumorigenesis, anchorage-independent growth was estimated. Cells undergoing apoptosis in response to serum depletion and cell detachment were treated with LBaP for 2 h, washed twice with serum-free DMEM, and cultured without serum for 24 h. Without the treatment with LBaP, over 80% of cells died. As almost cells died in further culture with serum-containing DMEM, LBaP-untreated cells could not be used for next induction of apoptosis (serum depletion and cell detachment). LBaP-treated cells were further cultured in serum-containing DMEM for 1 week and exposure to LBaP after the induction of apoptosis was repeated. This series of treatments was carried out three times. The colony-forming ability of the cells which survived apoptosis by LBaP was examined in soft agar. A double layer culture technique was carried out employing a bottom layer of 0.6% noble agar and a top layer of 0.3% agar in DMEM supplemented with 10% CS. Normal NIH3T3 cells and the cells which survived after treatment with LBaP were plated at 1×10^4 cells/60 mm dish. After 3 weeks, the colonies were photographed under a microscope and the numbers of colonies with diameter of 50–150 μm and of over 150 μm were counted, respectively.

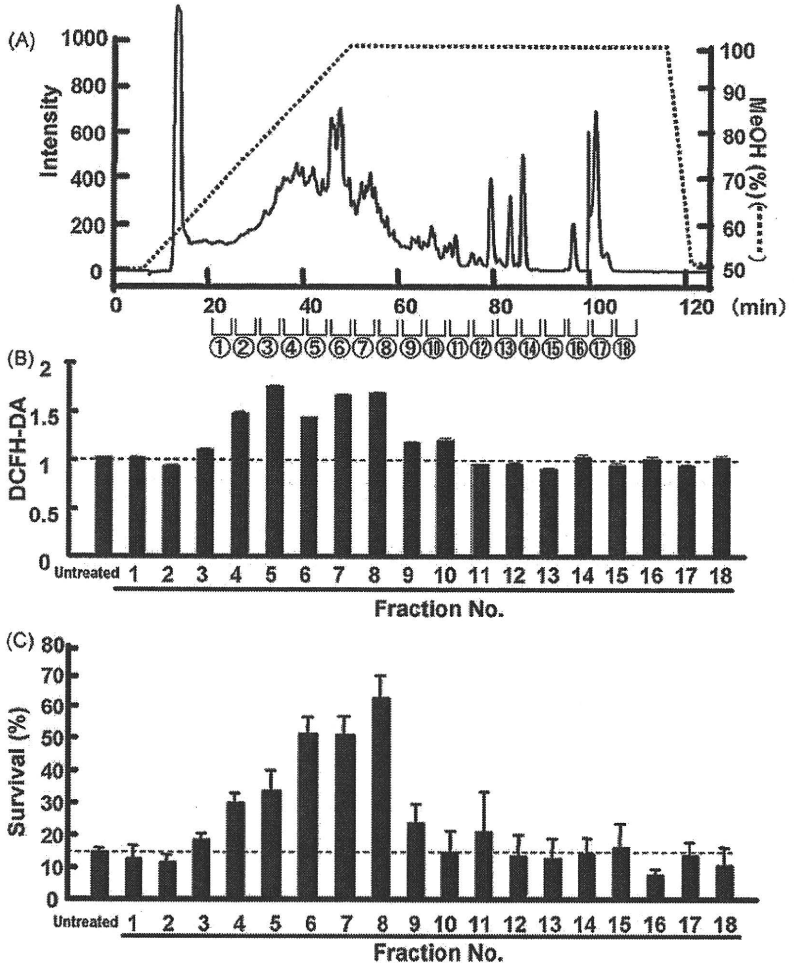

Fig. 5. Suppression of cell death and intracellular peroxidation by fractionated LBaP. BaP was exposed to SSL for 48 h and fractionated by HPLC. (A) HPLC chromatogram and fractionated pattern of LBaP. (B) Intracellular peroxidation after treatment with fractionated LBaP. (C) Survival after treatment with fractionated LBaP. LBaP was separated by HPLC and the fractionated LBaP was concentrated by evaporation. The fractionated LBaP, prepared at 120 μM as the dose of original LBaP, was treated and survival of NIH3T3 cells was determined as described in the legend to Fig. 1.

2.9. Tumorigenesis assay in vivo

Specific pathogen-free female 7-week-old BALB-c *nu/nu* nude mice (Japan SLC Inc., Shizuoka, Japan) were used for the tumorigenesis assay. Normal NIH3T3 cells and the cells that survived apoptosis after treatment with LBaP three times were suspended in PBS (5×10^6 cells/ml). The cells (10^6 cells/200 μl) were injected subcutaneously into the left leg. Tumor volume (mm^3) was estimated from femur length, groin width, and groin thickness every three days. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

2.10. HPLC analysis of LBaP

LBaP was analyzed using a 1100 series HPLC system (Agilent Technologies, Palo Alco, CA) with a UV detector. The separation was carried out on a reversed-phase column (Wakosil-PAHs, 4.6 mm i.d. × 250 mm, Wako Pure Chemical, Japan). The column temperature was 40 °C. The separation was conducted as described elsewhere [23]. The mobile phase solvent A was 5 mM ammonium acetate/0.02% formic acid in distilled water. Solvent B was 5 mM ammonium acetate/0.02% formic acid in methanol. The elution program was as follows: A:B = 50:50 (v/v) held for 5 min, followed by a linear gradient to 100% B in 45 min, held for 65 min, then back to initial conditions and equilibration for 5 min. The flow rate was 0.2 ml/min. The eluates were monitored for absorbance at 254 nm.

2.11. Statistics

All experiments were repeated two or three times. Values are means ± S.D. ($n = 3$–6). Data were analyzed by one-way ANOVA followed by Dunnett's t test for comparison of multiple samples with a control sample. Statistical significance is reported when $p < 0.05$, $p < 0.01$, and $p < 0.001$ and expressed as one, two, or three asterisks, respectively.

3. Results

3.1. Inhibition of cell death by LBaP

About 80% of NIH3T3 cells were dead 24 h after loss of cell adhesion and serum depletion, which was effectively inhibited by treatment with LBaP produced by exposure to SSL for 48 h (Fig. 1A). The inhibition was dose-dependent, the most effective dose being 40 μM. On the other hand, BaP (sham-exposure) did not have the same effect. LBaP exposed to SSL for 36–60 h markedly inhibited cell death (Fig. 1B). A longer exposure (72–96 h) gradually attenu-

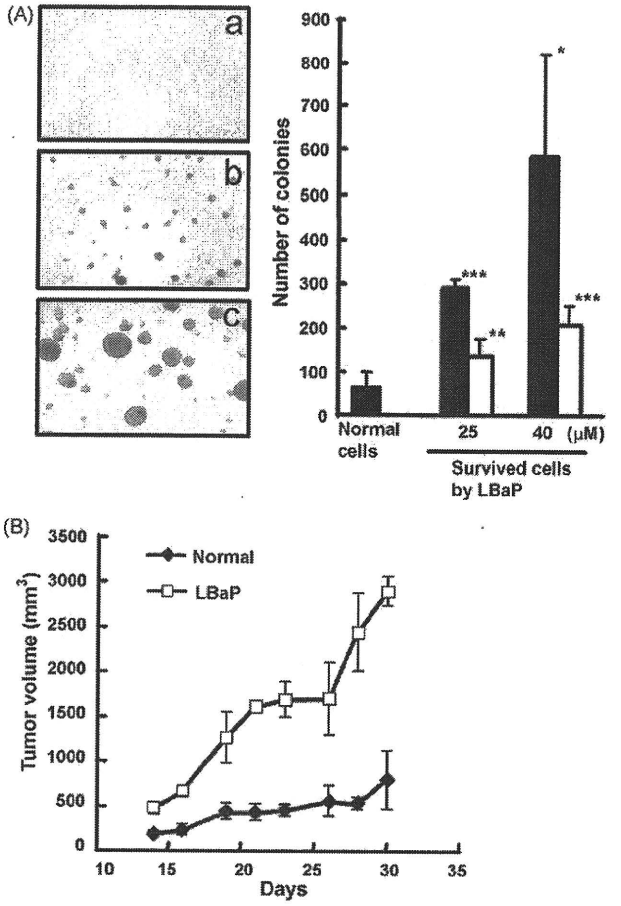

Fig. 6. Tumorigenesis of cells that survived apoptosis after treatment with LBaP. (A) Colony formation in soft agar. Normal NIH3T3 cells and the cells that survived apoptosis after treatment with LBaP (25 or 40 μM) three times were plated in soft agar (1×10^3 cells/dish) and cultured for 3 weeks. Micrographs: a: normal NIH 3T3 cells, b, c: cells that survived after treatment with LBaP (b) 25 μM and (c) 40 μM, respectively. Black column: colonies with diameter 50–150 μm, white column: colonies with a diameter greater than 150 μm. Values are means ± S.D. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Transplantation in nude mice. Normal NIH3T3 cells and the cells that survived apoptosis after treatment with LBaP (40 μM) three times, were suspended in PBS (5×10^6 cells/ml). The cells (10^6 cells/200 μl) were injected subcutaneously into the left leg of BALB-c *nu/nu* nude mice. Tumor volume (mm^3) was estimated from femur length, groin width and groin thickness every three days.

ated the effect (data not shown). We treated cells with LBaP for 2 h in all experiments because the effect of inhibition of cell death by LBaP was not different between treatment for 1 and 2 h (data not shown). The NIH3T3 cell line was used because of its suitability for assaying carcinogenesis, but the suppression of cell death by LBaP was also observed in other cell lines (Supplementary Fig. 1).

3.2. Inhibition of apoptosis by LBaP

To clarify that the inhibition of cell death by LBaP was due to the inhibition of apoptosis, the effects of LBaP on apoptotic factors: chromatin condensation, DNA fragmentation, and caspase activation were examined (Fig. 2). Fig. 2A shows images and percentages of chromatin condensed cells. Chromatin condensation induced by cell detachment and serum starvation was significantly attenuated by LBaP treatment, but not by treatment with BaP itself. DNA fragmentation was also attenuated by LBaP (Fig. 2B). As described in a previous paper [19], large fragments (50–150 kbp), not oligonucleosomal fragments (123 bp) of DNA were detected 12 h after cell detachment and serum depletion. The fragments disappeared after treatment with LBaP, dependent on the concentration of LBaP.

Caspase-3 is responsible for cleaving cellular proteins during apoptosis. Procaspase-3 is activated by proteolytic cleavage of the 32 kDa precursor at aspartic acid residues to generate an active heterodimer of 17 and 12 kDa subunits. Cell detachment and serum-free conditions induced the accumulation of processed products, which decreased after the treatment with LBaP (Fig. 2C). Caspase-3 activity detected by the degradation of a fluorogenic substrate was also inhibited by the treatment with LBaP, consistent with the proteolysis (Fig. 2D). On the other hand, high concentrations of LBaP (over 70 μM) reversed the suppression of apoptosis. This might be attributable to the toxicity of an excess of LBaP. These results suggested that certain doses of LBaP prevent apoptosis induced by cell detachment plus serum depletion, but the effect was not shown by BaP itself or an excess of LBaP.

3.3. Relationships between ROS production from LBaP and antiapoptosis

LBaP induced intracellular oxidation dose-dependently, implying the production of ROS (Fig. 3A). Fig. 3B shows intracellular oxidation dependent on the period of exposure to SSL. The intracellular oxidation correlated with the inhibition of cell death (survival). In addition, the antioxidant NAC significantly decreased the LBaP-induced antiapoptotic effect (Fig. 3C), suggesting that ROS produced from LBaP play a part in suppressing apoptosis.

The color change of the BaP solution with increasing duration of exposure to light was consistent with a chemical change in the BaP. The BaP solution, which was transparent and colorless, became gradually yellow to brown with exposure to SSL (Fig. 4A). HPLC indicated that BaP was broken down into compounds having higher polarity (Fig. 4B). The largest amounts of these compounds (eluted time: 20–100 min) were observed 48 h after exposure, which was consistent with the timing of the peak in the antiapoptotic effect and in the production of ROS in Figs. 1–3. Longer exposure to SSL resulted in further degradation of the newly produced compounds and almost all those that eluted in 20–100 min under HPLC-based separation disappeared.

3.4. ROS production and antiapoptosis of fractionated LBaP

The LBaP produced by exposure to SSL for 48 h was fractionated by HPLC (Fig. 5A). Intracellular oxidation was observed when the cells were treated with fractions 4–8 (Fig. 5B). Consistent with the intracellular oxidation, antiapoptotic activity was enhanced (Fig. 5C). These results suggested that several photoproducts (not just a single product) could produce an antiapoptotic effect.

BaP-quinones (BaP-1,6-dione and BaP-3,6-dione) have been identified as photomodified BaPs [2,6]. Although BaP-1,6-dione and BaP-3,6-dione had a similar antiapoptotic effect, they were less effective than the LBaP produced by exposure to SSL for 48 h (Supplementary Fig. 2A). In addition, the retention time in the HPLC analysis was different from that of the fractions having an antiapoptotic effect (35–60 min) (Supplementary Fig. 2B).

From the analysis by LC/ECI/MS/MS, two compounds were suspected to be involved in Fraction 7 (Supplementary Fig. 3). As shown in Supplementary Fig. 3A, a number of positive ionized peaks of degraded products of BaP were revealed. The MS spectral pattern of the peak at 50.44 min (Supplementary Fig. 3B) suggested that both BaP-4,5-dihydrodiol and 2-hydroxy-BaP-1,6-dione were mixed, identical to the spectra of Ref. [4] (Supplementary Fig. 3C).