ミン，DMAP 触媒にて行った．反応溶液を LCMS により分析した結果を Figure 3 に示す．無水酢酸の当量数を 5 および 10 当量にて検討したところ，生成物の MS ピークを確認したが，反応は完結せず，ほとんどが原料回収となった．さらに，反応時間を延長したところ，20 時間では，生成物が消失する結果となった（Figure 4）．8 位アセチル基の周囲は嵩高い構造に囲まれていることから，試薬との反応性が低いことが考えられる．そこで 6 位に Bn 基を導入することで化合物の物性や N の反応性が変化することを期待し，検討を行った．6 位 Bn 保護体を既報にしたがって調整し，無水酢酸を 5 当量用いて反応時間の検討を行ったが，18 時間までは目的化合物の反応が進むことを確認したが，42 時間ではその生成物の量が減少した（Figure 5）．また，一方で 2 位脱 DMTr 体や脱リボース体等の副生成物の生成が認められた．これは，過剰に加えた無水酢酸から生じた酢酸の影響によるものだと考えられる．より詳細な検討を続けることで，目的化合物が得られる可能性が示唆されたが，反応が完了しないことや副生成物の生成，スケールアップが難しいこと考慮し，本化合物の合成は断念することとした．

2. MeIQx 付加体の合成

ヘテロサイクリックアミン（Heterocyclic amine，HCA）類は食品中の焦げに含まれる化学物質であり，これらが DNA アダクトを形成することが知られている（Figure 6）[3]．IQ[4]や PhIP[5]の DNA アダクトおよびそのホスホロアミダイト体についてはすでに合成法が報告されていることから，これまでに報告例のない MeIQx に着目し，その DNA アダクトおよびホスホロアミダイト体を合成することとした．本研究ではより効率良い合成法を検討するために各種保護基の検討を行った．

1) N2 無保護合成の検討

Rizzo らのグループ[4]は 3',5'位に環状のシリル系保護基を導入し，2 位のアミノ基を無保護の状態で 8 位にアミノ基を導入することに成功している（Figure 7）．すなわち，2 位のアミノ基は反応性が極めて低く，Buchwald-Hartwig 反応の条件では反応しないことを示唆している．そこで本研究では Scheme 2 に示す合計 7 ステップの合成ルートを計画した．そこで，化合物 **3** から **12** を合成する際に，2 当量 MeIQx，0.1 当量 $Pd_2(dba)_3$，1 当量 xantphos，2 当量 Cs_2CO_3，溶媒 DMF を用いて microwave にて 100℃，12 時間で反応させたところ，約 50%程度反応が進むことを確認した（Figure 8）．この反応液を後処理し，順相シリカゲルカラムクロマトグラフィーにて精製作業を行ったところ，原料と生成物の溶出位置が重なり，生成物を単離することができなかった（data not shown）．そこで 2 位アミノ基（N2）を DMTr で保護した化合物（Scheme 2，化合物 4）を用いて反応を行ったところ，原料と生成物の溶出位置は大きく異なったことから，今後の合成では N2 は保護した状態で進めることとした（Scheme 3）．

2) MeIQx 付加体の合成

合成ルートを Scheme 3 に示す．DMTr 基の導入と脱保護のため，Scheme 2 に比べて 2 ステップ長くなった．化合物 **3** に対し，ピリジン溶液中にて DMTrCl を反応させ **4** を収率 73%で合成した．次に Buchwald-Hartwig 反応にて **17** を得た．反応条件を検討した結果を Table 1 に示す．溶媒として 1,4-dioxane を用いた場合，反応が 50%程度しか反応が進行しなかった．そこで溶媒を THF に変更したところ，反応効率および収率の向上が認められた．反応効率には化合物の溶媒への溶解性が大きく影響するのだと考えられる．次に TCA により脱 DMTr し **18** を 61%の収率で得た．さらに DMF-DMA により **13** を 98%の収率で得た．

D. 結論

本研究では，オリゴ DNA を供給するために，DNA アダクトのホスホロアミダイト体を合成す

ることを目的として研究を行った．当初計画していたdG-C8-AAFは，8位アセチル基の導入に難航した．8位は周囲を嵩高い構造に囲まれており，アセチル基は容易に加水分解されると考えられる．したがって，試薬中，反応溶液中に含まれる水分子を厳密に取り除く等の処理が必要であると考えられる．残念ながらdG-C8-AAFの最終生成物を得ることはできなかったが，その検討過程において，6位保護基が必要ないことを明らかにすることができた．6位はBn基で保護することが一般的であるが，その脱保護条件は一定ではなく，化合物の構造に応じて様々な条件を検討する必要があった．6位保護基を省略することで，全体の収量向上につながることが期待される．

MeIQx付加体に関しては，N2の無保護反応についても検討した．反応は50%程度進行（反応時間12時間）したが，精製時，原料と生成物の溶出位置が一致していたことから，生成物の単離には至らなかった．反応進行率が100%になる条件を見出すかあるいは原料と生成物の溶出位置が異なる化合物の場合はそのような問題は生じないと考えられる．MeIQx以外の付加体を合成する際に再度検討したい．

MeIQxを基質としたBuchwald-Hartwig反応について検討し，反応溶媒にTHFが適していることを明らかとした．その条件を用いてMeIQx付加体の合成に成功した．今後，ホスホロアミダイト体の合成を進め，オリゴDNAの合成を行う予定である．

E. 参考文献

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F. 健康危機情報

特になし．

G. 研究発表

1) 特に無し

H. 学会発表

1) 特に無し

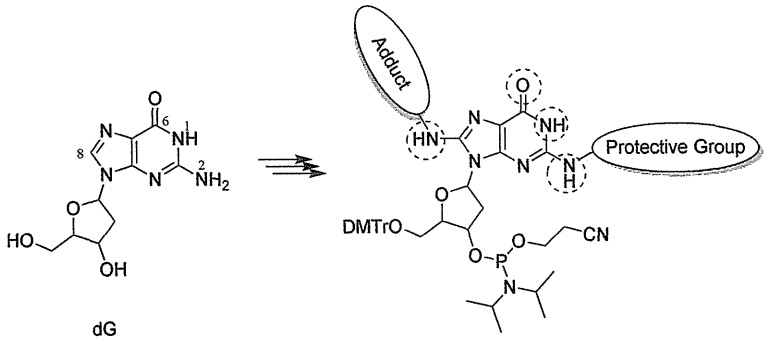


Figure 1 dG および, ホスホロアミダイト体の構造

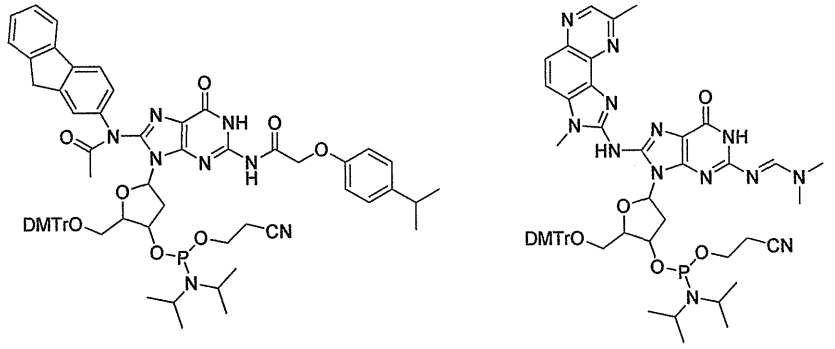
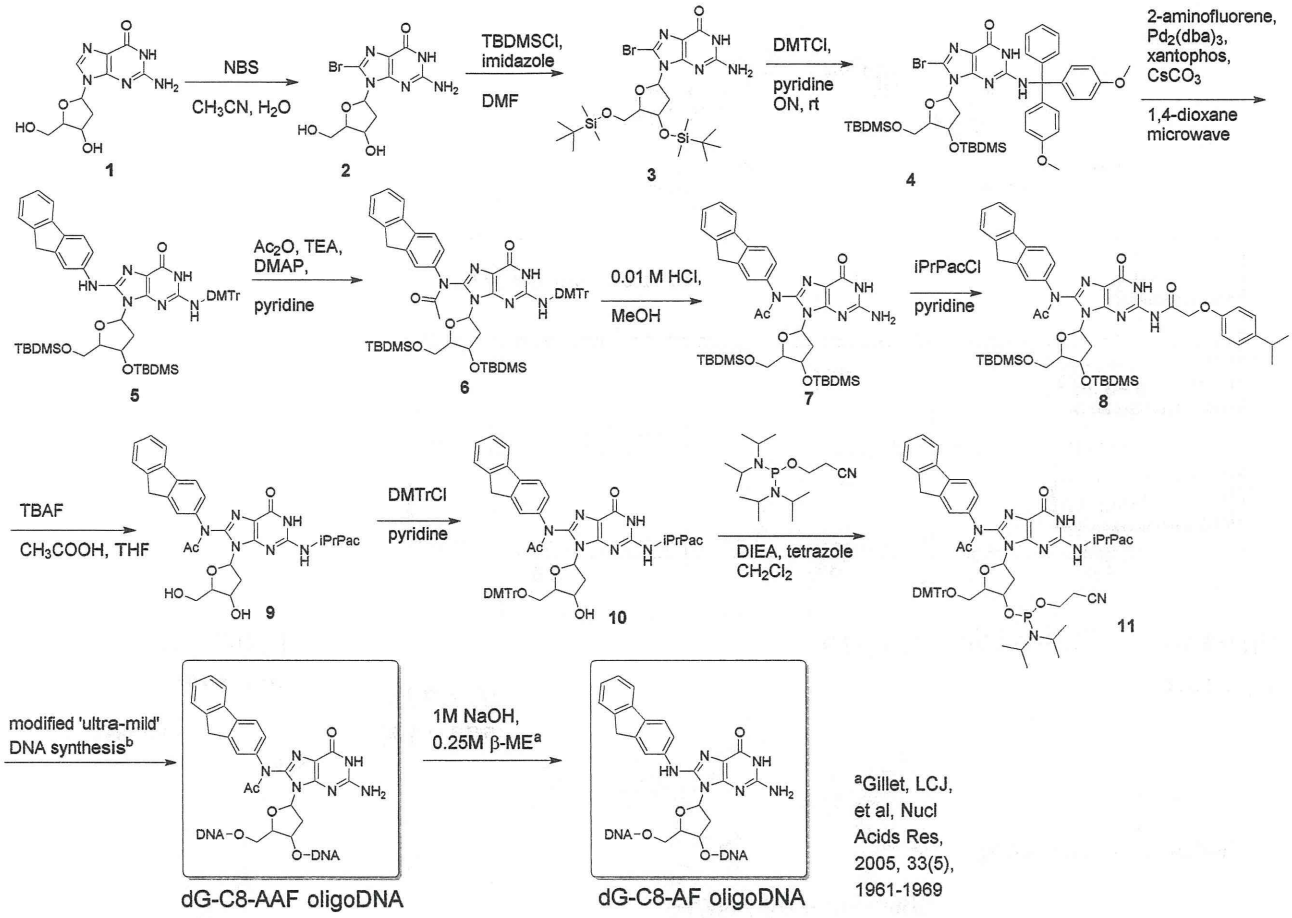


Figure 2　本研究で合成を試みた DNA のアダクト(ホスホロアミダイト体)の構造



Scheme 1 dG-C8-AAF の合成ルート

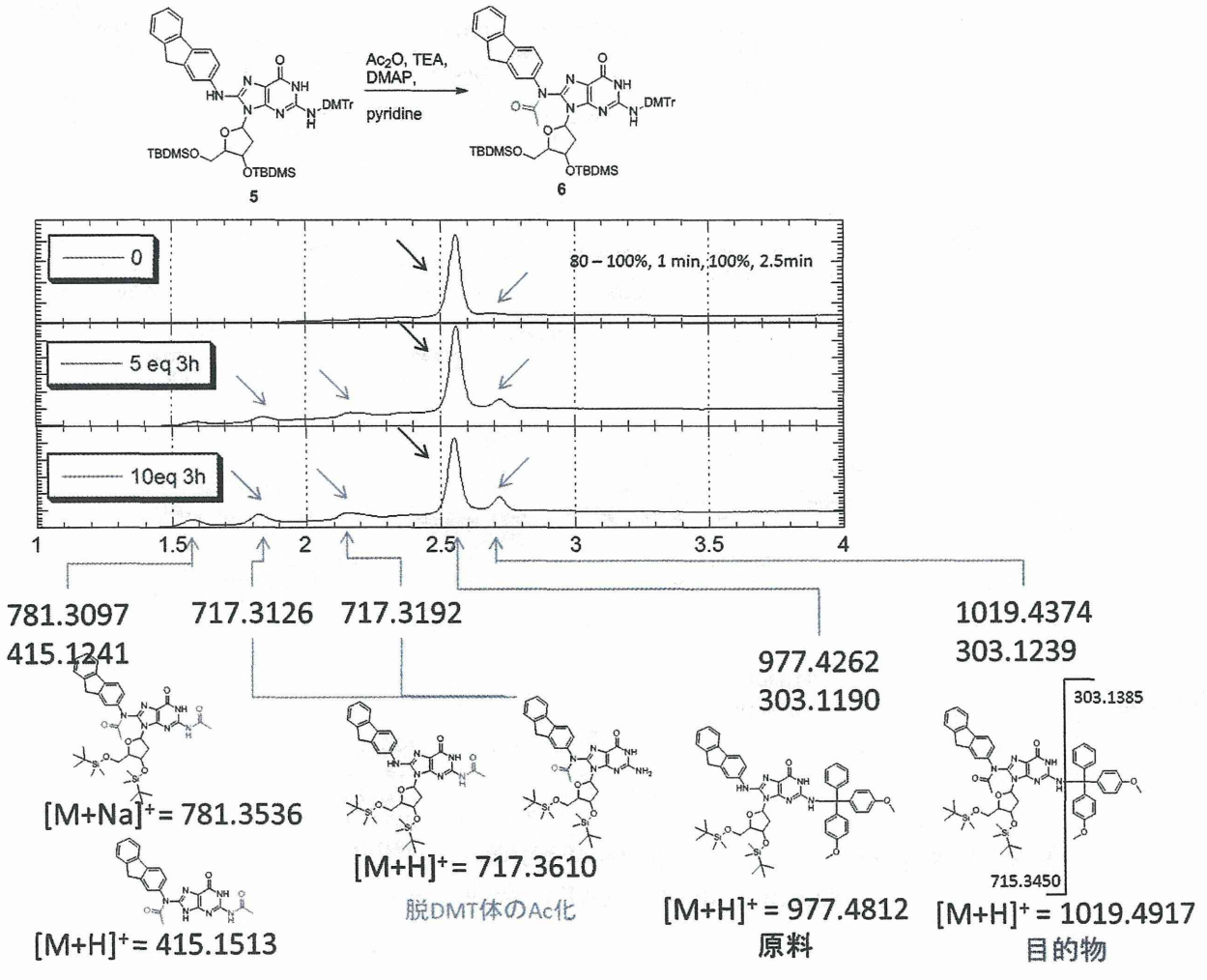


Figure 3. dG-C8-AAF 合成における 8 位 Ac 化の試薬濃度の検討. 反応スキームを上段に示した. 中段は UV 254 nm のクロマトグラムを示した. 検出された MS ピークから推定される化合物の構造式を下段に示した.

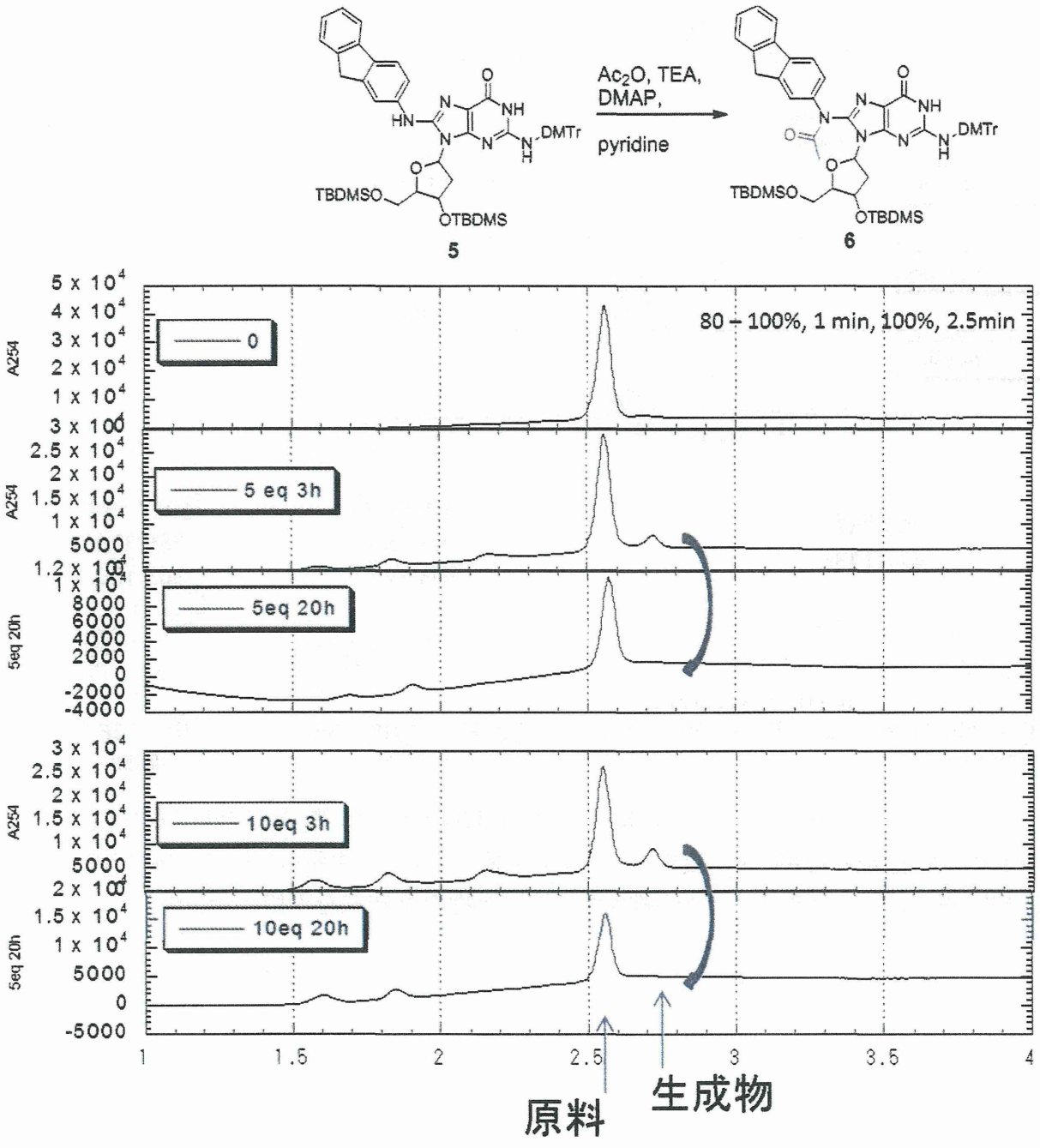


Figure 4. dG-C8-AAF 合成における 8 位 Ac 化の反応時間および試薬濃度検討. 反応スキームを上段に示した. 中段はピリジン 5eq での反応時間検討結果, 下段は 10eq での検討結果であり, それぞれ UV 254 nm のクロマトグラムを示した. MS から生成物および原料の保持時間を特定した.

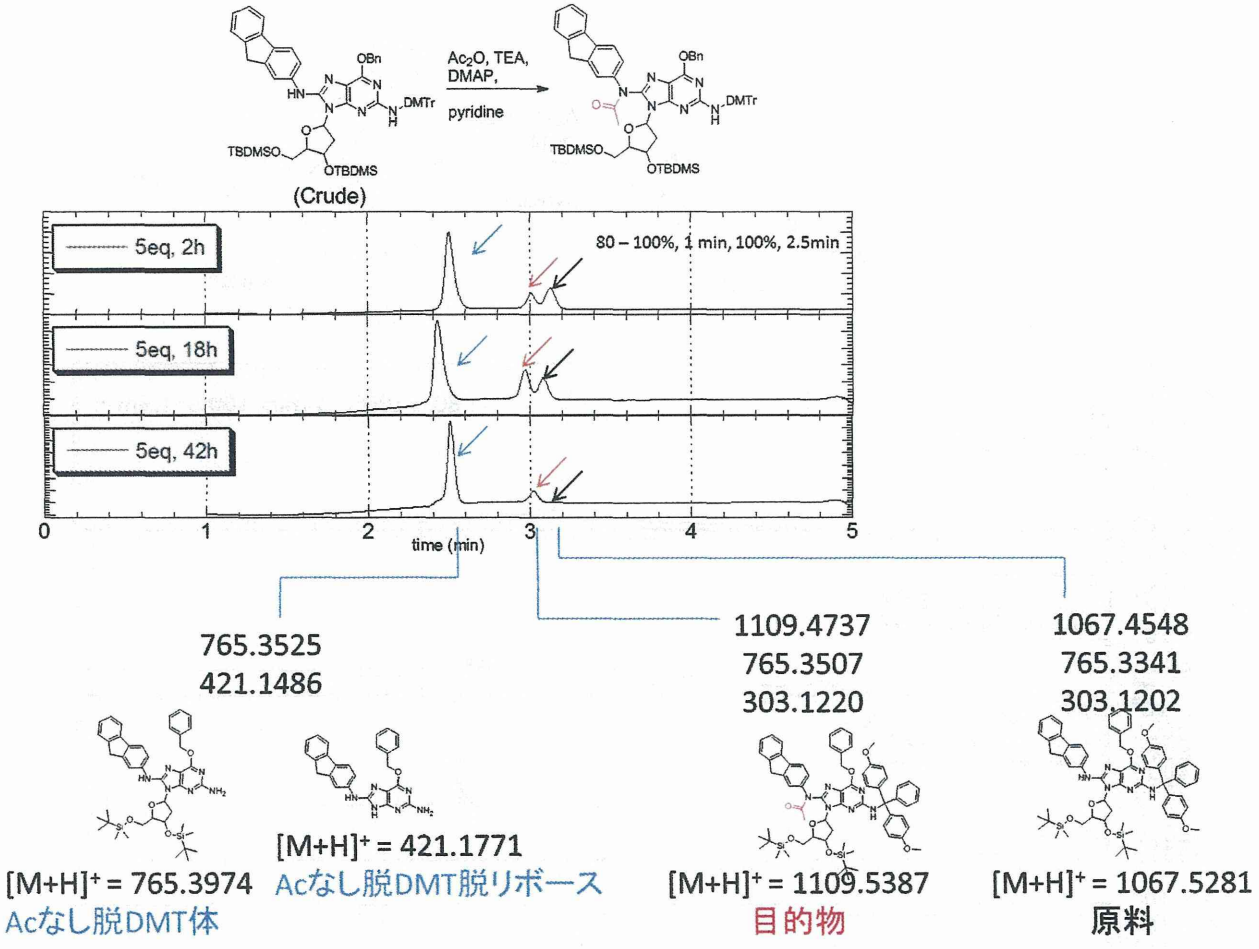


Figure 5　dG-C8-AAF 合成における 8 位 Ac 化の反応時間および試薬濃度検討. 原料として 6 位が Bn 保護された化合物を用いた. 反応スキームを上段に示した. 中段はピリジン 5eq での反応時間検討結果. 検出された MS ピークから推定される化合物の構造式を下段に示した.

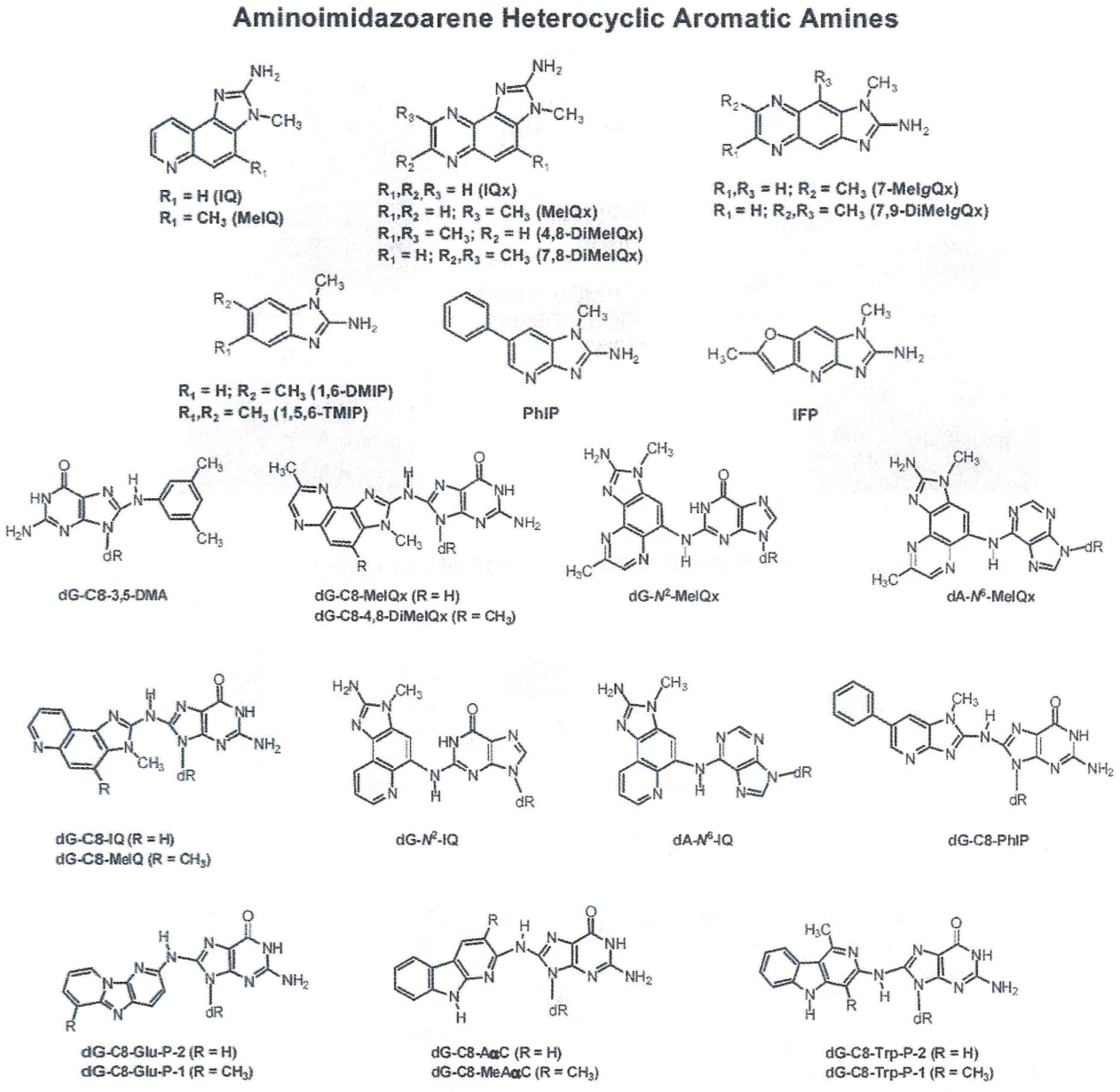


Figure 6　食品中に含まれるヘテロサイクリックアロマティックアミンの構造と，DNA アダクトの構造.

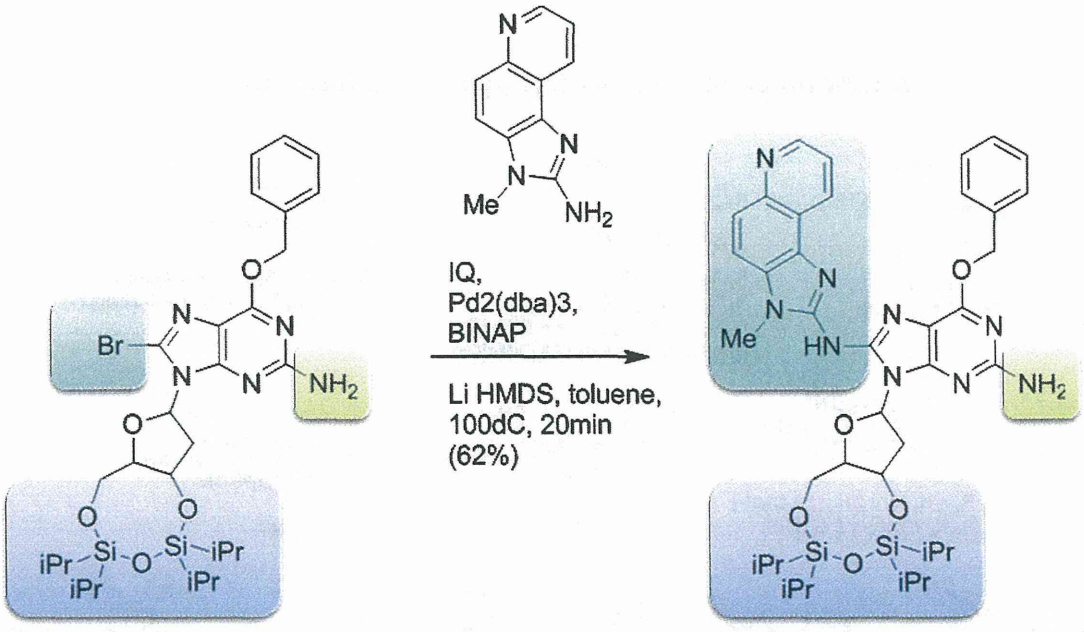
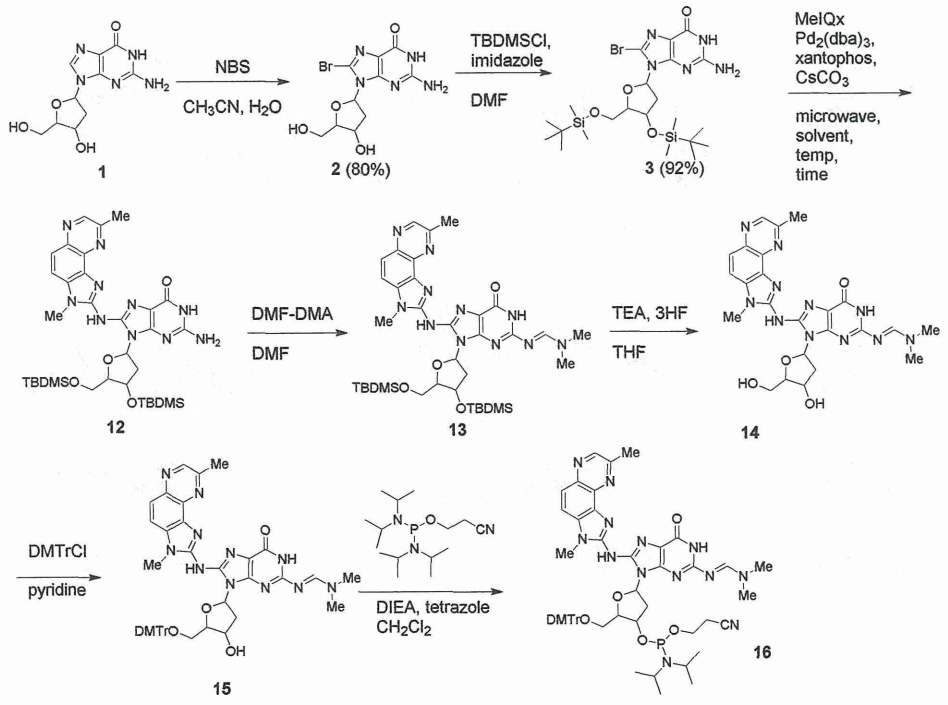


Figure 7　2 位無保護条件における Buckwald-Hartwig 反応



Scheme 2　dG-C8-MeIQx アダクトの合成スキーム.

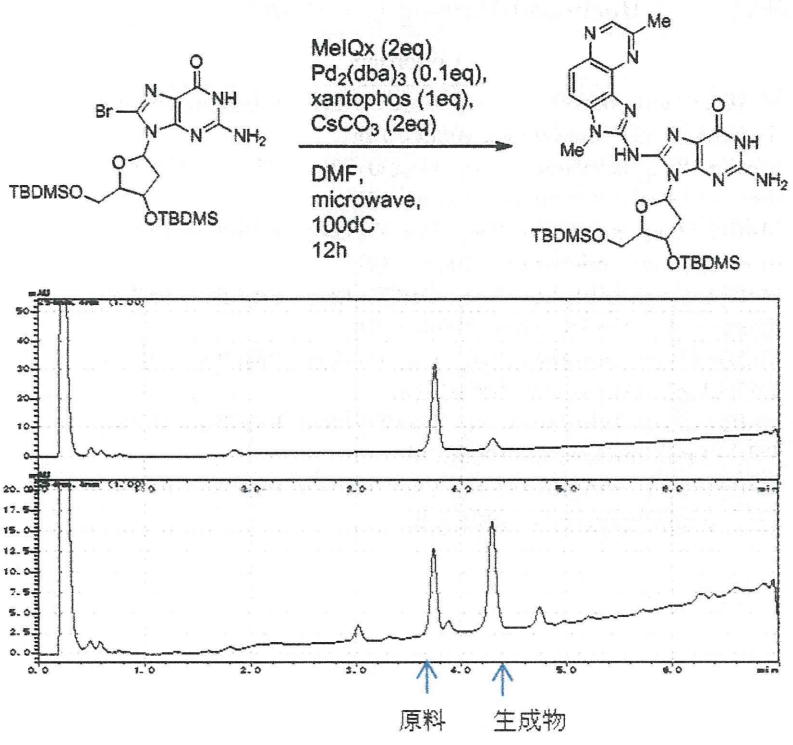
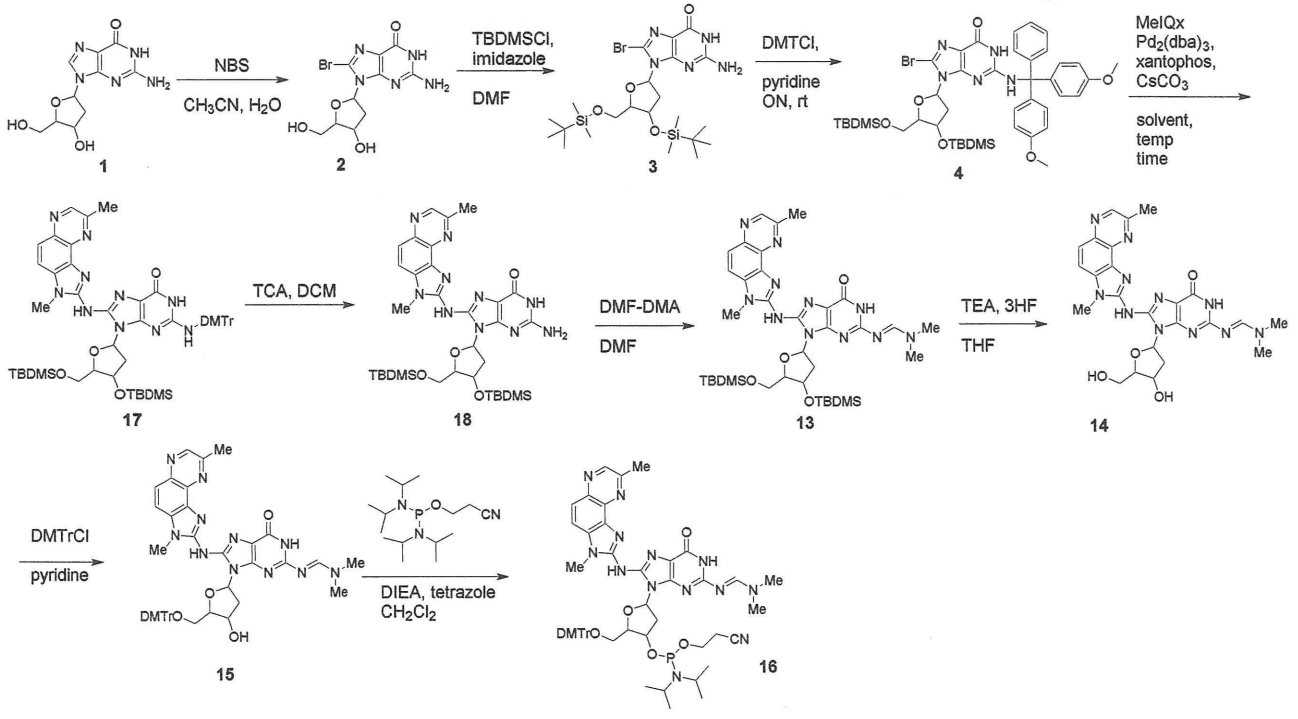


Figure 8　化合物 12 の合成，反応液を LCMS により分析した．UV 254 nm のクロマトグラムを示す．MS から生成物および原料の保持時間を特定した．



Scheme 3　dG-C8-MeIQx アダクトの合成スキーム(改)

Table 1　MeIQx を基質とした Buchwald-Hartwig 反応の結果

Entry	Scale	Condition	Yield
1	0.1 mmol	MeIQx (2 eq), xantphos (1 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.14 eq), dioxane (3 ml), microwave, 100dC, 15h	22% (50% complete)
2	0.1 mmol	MeIQx (2 eq), xantphos (1 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.14 eq), dioxane (3 ml), microwave, 130dC, 15h	28% (50% complete)
3	0.05 mmol	MeIQx (2 eq), xantphos (1 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.1 eq), dioxane (3 ml), microwave, 100dC, 12h	42% (50% complete)
4	0.1 mmol	MeIQx (2 eq), xantphos (2 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.2 eq), dioxane (3 ml), microwave, 100dC, 15h	dcomp
5	0.05 mmol	MeIQx (2 eq), xantphos (1 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.12 eq), THF (3 ml), microwave, 100dC, 12h	32%
6	0.05 mmol	MeIQx (2 eq), xantphos (1 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.2 eq), THF (3 ml), microwave, 100dC, 6h	58%
7	0.1 mmol	MeIQx (2 eq), xantphos (1 eq), Cs_2CO_3 (2eq), $Pd_2(dba)_3$ (0.2 eq), THF (5 ml), microwave, 100dC, 6h	63%

別添 5

III.　研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Honma M	Evaluation of the *in vivo* genotoxicity of Allura Red AC (Food Red No. 40).	Food and Chemical Toxicology	84	270-275	2015
Kanemaru Y, Suzuki T, Niimi N, Grúz P, Matsumoto K, Adachi N, Honma M, Nohmi T.	Catalytic and non-catalytic roles of DNA polymerase κ in the protection of human cells against genotoxic stresses.	Environ Mol Mutagen	56	650-662	2015
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Sugiyama, K., Takamune, M., Furusawa, H., Honma, M	Human DNA methyltransferase gene-transformed yeasts display an inducible flocculation inhibited by 5-aza-2′-deoxycytidine	Biochem. Biophys. Res. Commun.	456	689-694	2015
Ishino K, Kato T, Kato M, Shibata T, Watanabe M, Wakabayashi K, Nakagama H, Totsuka Y.	Comprehensive DNA adduct analysis reveals pulmonary inflammatory response contributes to genotoxic action of magnetite nanoparticles.	Int J Mol Sci.	16(2):	3474-92.	2015

Komiya M, Fujii G, Miyamoto S, Takahashi M, Ishigamori R, Onuma W, Ishino K, Totsuka Y, Fujimoto K, Mutoh M.	Suppressive effects of the NADPH oxidase inhibitor apocynin on intestinal tumorigenesis in obese KK-Ay and Apc mutant Min mice.	Cancer Sci.	106(11)	1499-505	2015
Misaki K, Takamura-Enya T, Ogawa H, Takamori K, Yanagida M	Tumour-promoting activity of polycyclic aromatic hydrocarbons and their oxygenated or nitrated derivatives	Mutagenesis	31(2)	205-213	2016
Takamura-Enya T, Tokutake M	Novel speciation analysis of copper in river water: observation of soluble anionic copper–ligand complexes	Limnology.	17	117-125	2016

IV.　研究成果の刊行物・別刷

Food and Chemical Toxicology 84 (2015) 270–275

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Evaluation of the *in vivo* genotoxicity of Allura Red AC (Food Red No. 40)

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ABSTRACT

Allura Red AC (Food Red No. 40) is a red azo dye that is used for food coloring in beverage and confectionary products. However, its genotoxic properties remain controversial. To clarify the *in vivo* genotoxicity, we treated mice with Allura Red AC and investigated the induction of DNA damage (liver, glandular stomach), clastogenicity/anuegenicity (bone marrow), and mutagenicity (liver, glandular stomach) using Comet assays, micronucleus tests, and transgenic gene mutation assays, respectively. All studies were conducted in accordance with the Organization for Economic Co-operation and Development (OECD) guideline. Although Allura Red AC was administered up to the maximum doses recommended by the OECD guideline, no genotoxic effect was observed in any of the genotoxic endpoints. These data clearly show no evidence of *in vivo* genotoxic potential of Allura Red AC administered up to the maximum doses in mice.

1. Introduction

The azo dye Allura Red AC (Food Red No. 40) has been approved as a food additive in the USA, Europe, and Japan and in many other countries. The safety of Allura Red AC was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1980 (JECFA, 1980) and then by the EU Scientific Committee for Food (SCF) in 1984 and 1989 (SFC, 1984, 1989). Based on available *in vivo* and *in vitro* genotoxicity studies, both committees concluded that Allura Red AC did not show any genotoxic potential. The committees also concluded that there was no evidence of carcinogenicity of Allura Red AC and established an Acceptable Daily Intake (ADI) of 0–7 mg/kg/day. However, Tsuda et al. performed a Comet assay in mice and showed that Allura Red AC induced significant increases in the migration of nuclear DNA in glandular stomach and colon tissues without causing general cytotoxicity (Tsuda et al., 2001). Consequently, the safety of Allura Red AC was re-evaluated by the panel of the European Food Safety Authority (EFSA) in 2009 (EFSA, 2009). Considering negative carcinogenicity and other genotoxicity studies of Allura Red AC, however, the biological significance of the Comet assay results is uncertain. The data from the study by Tsuda et al. (2001) were also used in a more comprehensive study on the Comet assay of a broad range of food additives (Sasaki et al., 2002). This study was not further discussed because it did not present any new data (EFSA, 2009).

To further clarify the controversies surrounding the report by Tsuda et al. (2001), we evaluated *in vivo* genotoxicity of Allura Red AC in mice using multiple genotoxicity tests consisting of Comet assays (liver, glandular stomach), micronucleus tests (bone marrow), and transgenic rodent (TGR) gene mutation assays (liver, glandular stomach), which can systematically assess the genotoxic potential of Allura Red AC inducing DNA damage, clastogenicity/aneugenicity, and mutagenicity, respectively. All studies were conducted in accordance with the Organization for Economic Co-operation and Development (OECD) guideline under Good Laboratory Practice (GLP) compliant conditions.

2. Materials and methods

2.1. Test chemicals and genotoxicity testing

The test chemical Allura Red AC [lot no. 060424, Chemical Abstracts Service Registry Number (CASRN) 25956-17-6] was supplied by San-Ei Gen F.F.I., Inc. (Osaka, Japan) with 87.5% purity. Allura Red AC was tested using Comet assays, micronucleus tests, and TGR assays, which were performed by the Food and Drug Safety Center, Hadano Institute, Kanagawa, Japan, the Safety Research Institute for Chemical Compounds, Hokkaido, Japan, and the BioSafety Research

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0278-6915/

Center, Shizuoka, Japan, respectively. All studies were performed under GLP compliant conditions in accordance with the OECD guideline. Animals were treated in accordance with regulations of the Animal Care and Use Committees of the laboratories, and the National Institute of Health Sciences (NIHS), Japan.

2.2. Comet assay

2.2.1. Animals and treatment

The Comet assay was conducted in accordance with TG 489 (*In vivo* mammalian Alkaline Comet Assay; OECD, 2014).

Male CD2F1 mice [CD2F1/Crlj (CDF1), SPF/VAF] were purchased at the age of 8 weeks from Charles River Laboratories, Japan. Animals were acclimatized for 8 days before the start of the treatments. Food (CE-2 pellet feed, CLEA Japan) and water were provided *ad libitum*. In total, 25 mice were used in experiments, including 5 mice in each of the 3 dose groups and 5 each in the positive and negative control groups.

The test chemical and the positive control (ethyl methanesulfonate; EMS, lot no. 125K1797, Sigma Aldrich, St. Louis, MO) were dissolved in physiological saline (Hikari Pharmaceutical, Japan) prior to the treatment of animals. Dosing formulations of the test chemical and the negative control substance were orally administered 2 times using stomach tubes and plastic syringes at 24 h and 3 h before tissue sampling. Dosing formulations of the positive control substance were orally administered once using stomach tubes and plastic syringes at 3 h before tissue sampling. The maximum dose (2000 mg/kg) was determined using preliminary toxicity tests (data not shown), and was the highest dose used in OECD TG489. Therefore, doses of 2000, 1000, and 500 mg/kg were tested.

2.2.2. Comet analyses

Mice were euthanized by exsanguination after intraperitoneal injection of pentobarbital sodium. The liver and stomach were removed from each mouse, and portions of the left lateral lobe of the liver were removed and washed in cold mincing buffer (Hank's balanced salt solution without calcium and magnesium) containing 20 mM Na_2EDTA and 10% (v/v) dimethyl sulfoxide until as much blood as possible had been removed. Stomach epithelia were collected from the surface of the glandular stomach by gently scraping 4–5 times. Samples of the liver lobe and surface epithelia from the glandular stomach were minced using a pair of fine scissors to release cells. The cells were then suspended in cold mincing buffer, were filtered through a Falcon Cell Strainer (50-μm pore, Becton, Dickinson and Company, Franklin Lakes, NJ) to remove lumps, and were analyzed using Comet assays according to previously described procedures (Burlinson et al., 2007; Hartmann et al., 2003; Speit et al., 2015). In brief, cells were embedded in 0.5% (w/v) low-melting point agarose gels (NuSieve GTG, Lonza, Allendale, NJ) on 2 slides for each organ. The slides were then immersed in chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (hydroxymethyl) aminomethane, 1% (v/v) Triton-X100, and 10% (v/v) dimethyl sulfoxide (pH 10) overnight in a refrigerator. After cell lysis, the slides were rinsed with cold purified water and randomly placed onto a platform in a submarine-type electrophoresis tank. Subsequently, electrophoresis solution containing 300 mM sodium hydroxide and 1 mM Na_2EDTA was added until all slides were completely covered with solution. The slides were then incubated for 20 min to allow the DNA to unwind, and were then electrophoresed for 20 min at a constant voltage to achieve approximately 0.7 V/cm. The electrophoresis current was adjusted to approximately 0.30 A by the addition of electrophoresis solution, and the electrophoresis solution was maintained below 10 °C during unwinding and electrophoresis. Subsequently, slides were immersed in cold neutralization buffer containing 400 mM Tris (hydroxymethyl) aminomethane (pH 7.5) for 20 min, were dehydrated by immersion in ethanol for 10 min, and were then dried and stored at room temperature until observation. Slides were stained with SYBR Gold solution (Life Technologies, Rockville, MD) and were observed by fluorescence microscopy with a blue extinction filter (excitation 460–490 nm, emission 515 nm) and a × 200 lens. Numbers of heavily damaged cells (hedgehogs) among 100 cells per tissue were manually determined using 2 slides, and the cells were separately counted using an image analyzer system (Comet assay IV, Perspective Instruments, Suffolk, England). For each tissue, 100 cells were analyzed using two slides. DNA damage was assessed according to percentages of tail DNA, which were calculated as the intensity of the Comet tail relative to the total intensity.

2.2.3. Statistics

Mean percentages of tail DNA were compared between each treatment group and the negative control group using Dunnett's multiple comparison test (1-tailed), and differences were considered significant when $p < 0.05$ and $p < 0.01$.

2.3. Micronucleus test

2.3.1. Animals and treatment

The micronucleus test was conducted in accordance with TG 474 (Mammalian Erythrocyte Micronucleus Test, OECD, 1997).

Male CD1 mice (Crlj:CD1) were purchased at 6 weeks of age from Charles River Laboratories, Japan and were acclimatized for 7 days before treatments. Food (CRF-1 pellet feed, Oriental Yeast, Japan) and water were provided *ad libitum*. Thirty mice were used for the experiment (6 mice per treatment, positive control, and negative control group).

The test chemical was dissolved in 0.5% carboxymethylcellulose sodium (CMC; lot no. 8108, Maruishi Pharmaceutical, Japan) solution prior to administration. As a positive control, Mitomycin C (MMC; lot no. 7K81, Kyowa Kirin, Japan) was dissolved in water for injection (Otsuka Pharmaceutical Factory, Japan). Dosing formulations of the test article and the negative control (0.5% CMC) were orally administered daily for 2 days with a 24-h interval using stomach tubes and plastic syringes. Dosing formulations of the positive control substance were administered in a single intraperitoneal injection using a disposable syringe fitted with a 25-G needle. The maximum dose (2000 mg/kg) was determined from the literature (JECFA, 1980) and was the highest dose used in OECD TG474. Therefore, doses of 2000, 1000, and 500 mg/kg were tested.

2.3.2. Micronucleus analyses

Mice were sacrificed by cervical dislocation at 23–24 h after the final treatment. Subsequently, femurs was removed and bone marrow cells were flushed from femurs into centrifuge tubes using calf serum (lot no. 1361699, Life Technologies, Japan). Cells were then resuspended in Dulbecco's phosphate-buffered saline (PBS, lot no. RNBD3941, Sigma–Aldrich, Japan) and were centrifuged, and supernatants were then removed. These procedures were performed twice, and then cells were fixed in 10% neutral-buffered formalin solution (for tissue fixative use: lot no. KPP6385, Wako Pure Chemical, Japan). After exchanging the fixative twice by centrifugation, cells were re-suspended in formalin solution, and the cell suspension was filtered through a cell strainer (pore size 35 μm, Becton, Dickinson and Company, Franklin Lakes, NJ). Fixed cell suspensions were then dropped onto cover slips and were immediately placed on acridine orange coated slides (lot no. SDE3454, Wako Pure Chemical, Japan). Two thousand polychromatic erythrocytes (PCEs) per animal were analyzed using a fluorescent microscope with a × 1000 lens, equipped with a blue

excitation filter and a barrier filter (BX50: BXFLA, Olympus, Japan), and numbers of micronucleated polychromatic erythrocytes (MNPCEs) were counted. To investigate the influence of the test substance on bone marrow cell proliferation, numbers of PCEs in a total of 500 erythrocytes were counted.

2.3.3. Statistical analysis

Frequencies of MNPCEs in treatment and positive control groups were compared with those in the negative control group using conditional binomial tests (Kastenbaum and Bowman test, upper-tailed significance level of 0.05).

2.4. TGR assay

2.4.1. Animals and treatment

TGR assay was conducted to assess the induction of gene mutations in the *cII* gene using Muta™ Mice (Jakubczak et al., 1996) in accordance with TG 488 (Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays; OECD, 2013).

Male Muta™ Mice (CD_2-LacZ80/HazfBR) were purchased at 9 weeks of age from Japan Laboratory Animals, Inc., Japan and were acclimatized for 11 days before treatments. Food (CRF-1 pellet feed, Oriental Yeast, Japan) and water were provided *ad libitum*. In total, 28 mice were used for the experiment (6 mice per treatment and negative control groups and 4 mice in the positive control group).

The test chemical was dissolved in water for injection (Otsuka Pharmaceutical Factory, Japan) prior to administration. The positive control 7, 12-dimethylbenz[a]anthracene (DMBA, lot no. FIN01, Tokyo Chemical Industry, Co., Ltd., Japan) was mixed with olive oil (lot no. PEL2965, Wako Pure Chemical Industries, Japan), and dosing formulations of the test substance and the negative control (water for injection) were orally administered once daily for 4 weeks using stomach tubes and plastic syringes. Dosing formulations of the positive control substance were administered by intraperitoneal injection once daily for 2 days using a disposable syringe fitted with a 25-G needle. The maximum dose (1000 mg/kg) was determined from the literature (JECFA, 1980), and it was the highest dose used in OECD TG488. Therefore, doses of 1000, 500, and 250 mg/kg were tested.

2.4.2. Detection of gene mutations

Mice were sacrificed by CO_2 asphyxiation 3 days after the last treatment. Subsequently, liver and stomach tissues were collected, stomach tissues were divided into forestomach and glandular stomach, and genomic DNA was extracted from liver and glandular stomach samples according to previously reported methods (Matsumoto et al., 2014).

DNA packaging was performed according to the instruction manual of Transpack (Stratagene, La Jolla, CA). In brief, DNA solutions (200–600 μg/mL) were gently mixed with the Transpack packaging extract and were incubated 2 times at 30 °C for 1.5 h; SM buffer containing NaCl, $MgSO_4 \cdot 7H_2O$, Tris–HCl (pH 7.5), and gelatin was then added. *Escherichia coli* were absorbed into phage solution at room temperature for 20–30 min. An appropriately diluted *E. coli* solution was mixed with LB top agar for titer plates, and selection plates were produced by mixing the remaining phage–*E. coli* solution with LB top agar containing P-gal (phenyl-β-D-galactoside, Sigma–Aldrich). Selection plates were then incubated overnight at 37 °C, and packaging was repeated to reach a total number of 300,000 plaques. Mutant frequencies (MFs) were calculated as follows: MFs = total numbers of plaques on selection plates/total numbers of plaques on titer plates.

2.4.3. Statistical analysis

Differences in MFs were analyzed for significance by the Conditional Binomial test (Kastenbaum and Bowman method: upper tailed significance level of 0.05). The results were evaluated as positive when the mutant frequency in the test substance treated group was significantly different from that in the negative control group.

3. Results

3.1. Comet assays

No deaths and no clinical signs of toxicity were observed in any

Table 2
The results of micronucleus test in bone marrow of CD1 mice after Allura Red AC treatment.

Compound	Dose(mg/kg)	No. of animal	% MNPCE[a]	% PCE
Control	0	5	0.14 ± 0.07	63.3 ± 8.1
Allura Red AC	500	5	0.20 ± 0.04	61.8 ± 5.1
	1000	5	0.14 ± 0.07	61.4 ± 9.1
	2000	5	0.17 ± 0.14	62.9 ± 5.0
MMC	1	5	3.66 ± 0.94**	56.0 ± 8.1

**:$p < 0.01$, significant difference from control (Kastenbaum and Bowman method, upper-trailed).
Control: negative control (0.5% Carboxymethylcellulose sodium, 10 ml/kg).
MMC: positive control (Mitomycin C, dose once a day, for 2 days, *i.p.*, 1 days after administration).
[a] Polychromatic erythrocytes possessing one or more than one micronuclei (MNPCEs) were counted.

Table 1
The results of Comet assay in liver and glandular stomach of CDF1 mice after Allura Red AC treatment.

Tissues & compound	Dose (mg/kg)	No. of animal	Hedge-hogs (%)	% Of DNA in tail		Olive tail moment		Tail length(um)	
				Average ± SD	Median	Average ± SD	Median	Average ± SD	Median
Liver									
Control	0	5	4.0	2.8 ± 6.1	1.0	0.9 ± 2.0	0.3	64.6 ± 13.3	63.5
Allura Red AC	500	5	4.2	2.7 ± 4.5	1.2	0.7 ± 1.3	0.3	62.4 ± 12.0	64.1
	1000	5	2.0	3.4 ± 4.6	1.9	0.9 ± 1.3	0.6	68.7 ± 11.7	67.9
	2000	5	4.6	3.8 ± 4.9	2.3	1.0 ± 1.5	0.6	65.1 ± 12.9	64.3
EMS	300	5	6.0	11.5 ± 7.4*	9.8	3.1 ± 2.2	2.6	73.5 ± 11.8	72.3
Glandular stomach									
Control	0	5	12.8	9.6 ± 10.7	5.5	2.8 ± 3.4	1.5	73.2 ± 13.4	71.5
Allura Red AC	500	5	8.4	5.2 ± 9.0	1.2	1.5 ± 2.7	0.3	64.7 ± 14.5	63.3
	1000	5	10.8	7.0 ± 10.0	2.6	2.1 ± 3.3	0.7	69.1 ± 15.6	66.9
	2000	5	15.0	8.0 ± 12.2	2.5	2.4 ± 3.9	0.7	73.5 ± 15.9	71.5
EMS	300	5	10.9	16.1 ± 12.8*	11.6	4.6 ± 4.0	3.1	79.1 ± 13.7	77.3

*:$p < 0.05$, significant difference from control (Dunnett's multiple comparison test, one tailed).
Control: negative control (physiological saline, 10 ml/kg).
EMS: positive control (Ethyl methanesulfonate, one time, *i.p.*, 3 h after administration).

Table 3
The results of TGR assay in liver of Muta™ Mouse after Allura Red AC treatment.

Compound	Dose (mg/kg)	Animal ID no.	Number of plaque forming units	Number of mutant plaques	Mutant frequency ($\times 10^{-6}$)	Mutant frequency ($\times 10^{-6}$) Average ± S.D.
Control	0	001	585,000	45	76.9	61.1 ± 15.6
		002	1,070,100	43	40.2	
		003	569,700	41	72.0	
		004	313,200	21	67.0	
		005	505,800	25	49.4	
Allura Red AC	250	101	505,800	40	79.1	67.9 ± 10.2
		102	570,800	38	66.6	
		103	718,200	40	55.7	
		104	426,600	33	77.4	
		105	576,900	35	60.7	
	500	201	598,500	34	56.8	64.9 ± 10.3
		202	729,900	48	65.8	
		203	605,700	45	74.3	
		204	649,800	49	75.4	
		205	478,800	25	52.2	
	1000	301	308,700	13	42.1	57.5 ± 10.8
		302	456,100	33	72.0	
		303	430,200	26	60.4	
		304	474,300	26	54.8	
		305	1026,700	60	58.3	
DMBA	20	401	537,300	28	52.1	75.0 ± 20.5*
		402	522,900	48	91.8	
		403	456,300	37	81.1	

*:$p < 0.05$, significant difference from control (Kastenbaum and Bowman method, upper-trailed).
Control: negative control (water, 10 ml/kg).
DMBA: positive control (7, 12-Dimethylbenz[a]anthracene, dose once a day, for 2 days, *i.p.*, 3 days after administration).

of the treatment groups. Comet assays of liver and glandular stomach tissues were performed (Table 1). As indicators of DNA damage, percentages of DNA in the tail, Olive tail moments, and tail lengths were measured. Numbers of heavily damaged cells (hedgehogs) were also determined but were excluded from analyses of Comet data. No significant differences in numbers of hedgehogs or DNA damages were observed in either organ between Allura Red AC-treated animals and negative control animals. In contrast, the positive control (EMS) group showed significant increases ($p \leq 0.05$) in percentages of DNA in Comet tails from both organs.

3.2. Micronucleus tests

No deaths and no clinical signs of toxicity were observed in any of the treatment groups. No differences in body weight were observed between control and treatment groups at the end of treatment (data not shown). In micronucleus tests (Table 2), no significant differences in MNPCEs were found between Allura Red AC treatment groups and the negative control group. The frequency

Table 4
The results of TGR assay in glandular stomach of Muta™ Mouse after Allura Red AC treatment.

Compound	Dose (mg/kg)	Animal ID no.	Number of plaque forming units	Number of mutant plaques	Mutant frequency ($\times 10^{-6}$)	Mutant frequency ($\times 10^{-6}$) Average ± S.D.
Control	0	001	1,183,500	56	47.3	60.5 ± 9.8
		002	909,900	62	68.1	
		003	1,057,500	69	65.2	
		004	850,500	45	52.9	
		005	957,600	66	68.9	
Allura Red AC	250	101	845,100	56	66.3	65.2 ± 6.4
		102	1,010,700	56	55.4	
		103	947,700	68	71.8	
		104	932,400	65	69.7	
		105	635,400	40	63.0	
	500	201	788,400	44	55.8	48.1 ± 8.2
		202	972,900	41	42.1	
		203	1,467,900	65	44.3	
		204	1,103,400	64	58.0	
		205	1,215,900	49	40.3	
	1000	301	1,042,200	51	48.9	50.8 ± 10.0
		302	553,500	22	39.7	
		303	1,511,100	70	46.3	
		304	1,359,900	71	52.2	
		305	689,400	46	66.7	
DMBA	20	401	864,900	50	57.8	66.7 ± 15.2
		402	843,300	49	58.1	
		403	1,186,200	100	84.3	

Control: negative control (water, 10 ml/kg).
DMBA: positive control (7, 12-dimethylbenz[a]anthracene, dose once a day, for 2 days, *i.p.*, 3 days after administration).

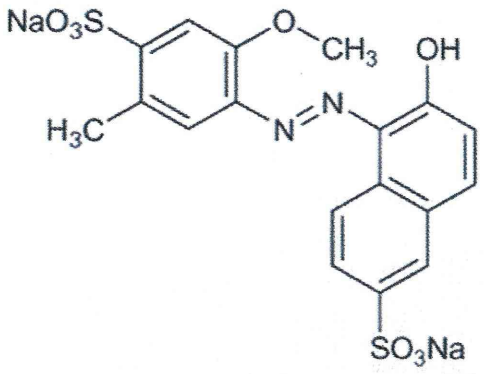

Fig. 1. Chemical structure of Allura Red AC (Food Red No. 40).

of PCEs, which offers an index of the influence of the test substance on bone marrow cells, did not differ between any of the treatment groups and the negative control group. In contrast, the frequency of MNPCEs in the positive control group was markedly increased ($p \leq 0.01$) in comparison with that in the negative control group.

3.3. TGR assay

No deaths and no clinical signs of toxicity were recorded in any of the treatment groups. In addition, body weight gains during treatment were the same in all treatment groups (data not shown). MFs of *cII* genes in liver and glandular stomach tissues from Allura Red AC-treated animals were not significantly higher than those in respective negative control animals (Tables 3 and 4). In contrast, the positive control DMBA significantly increased MFs in the liver ($p \leq 0.05$), and MFs were slightly but insignificantly induced in stomach tissues. However, these weak response may be adequate as positive control in TGR assay.

4. Discussion

Aromatic azo compounds such as Allura Red AC (Fig. 1) bear structures that have been associated with bacterial mutagenicity in Ames mutagenicity assays (Ashby and Tennant, 1988; Tennant and Ashby, 1991). However, no previous Ames mutagenicity assays have demonstrated the mutagenic potential of Allura Red AC (Brown et al., 1978; Fujita et al., 1995; Muzzall and Cook, 1979; Zeiger and Margolin, 2000). Similarly, no genotoxicity of Allura Red AC was demonstrated in *Saccharomyces cerevisiae* gene mutation assays, mouse heritable translocation assays, or a sex-linked recessive lethal test in *Drosophila melanogaster* (JECFA, 1980). There has been a few reports of genotoxic evaluation of Allura Red AC examined by OECD *in vivo* genotoxicity tests. Abramsson-Zetterberg and Ilback (2013) recently conducted flow cytometry-based *in vivo* mice micronucleus tests and demonstrated no induction of micronuclei in the bone marrow after single intraperitoneal injections of up to 2000 mg/kg Allura Red AC.

In contrast, Tsuda et al. (2001) conducted *in vivo* Comet assays to assess DNA damage after orally administering Allura Red AC to groups of male mice at doses of 0, 1, 10, 100, 1000, and 2000 mg/kg. Three hours after administration, significant increases in DNA damage were observed in glandular stomach tissues at doses of ≥100 mg/kg. In addition, significant differences in DNA damage in the colon were observed between treatment and control animals at doses of ≥10 mg/kg, with a dose–response relationship up to 100 mg/kg. In lung tissues, DNA damage was also slightly increased at a dose of 1000 mg/kg. Other organs, including the liver, kidney, bladder, brain, and bone marrow, exhibited no DNA damage at any dose. Moreover, histopathological examination revealed no necrotic and apoptotic effect on the colon and glandular stomach tissues, and the authors concluded that the observed DNA damage was not likely to produce severe cytotoxicity (Tsuda et al., 2001). This is only an original article demonstrating genotoxicity of Allura Red. Sasaki et al. (2002) also reported the results of *in vivo* Comet assay of broad range of food additives including Allura Red AC. However, these studies were conducted neither under GLP compliant nor in accordance with OECD guideline.

In the present study, we evaluated *in vivo* genotoxicity of Allura Red AC in mice using a series of genotoxicity tests consisting of Comet assay (liver, glandular stomach), micronucleus test (bone marrow), and TGR assay (liver, glandular stomach), which can systematically assess DNA damage, clastogenicity/aneugenicity, and mutagenicity, respectively. All studies were conducted in accordance with OECD guideline under GLP compliant condition. Allura Red AC was orally administered to mice up to the maximum dose recommended by the OECD guidelines; 1000 mg/kg/day for treatment period of 28 days (TGR assay) and 2000 mg/kg/day for treatment periods of 14 days or less (Comet assay and micronucleus test). No clinical signs of toxicity or weight gain were observed during the treatment period, indicating that the acute oral toxicity of Allura Red AC is very low in mice. Accordingly, no apparent cytotoxicity was observed in terms of numbers of hedgehogs in the liver and glandular stomach (Comet assay) or percentages of PCEs in the bone marrow (micronucleus test) at any of the doses tested. All genotoxic endpoints including Comet assays clearly indicated no genotoxic effect on the mice of any treatment of Allura Red AC, in contrast with the data reported by Tsuda et al. (2001).

Although Allura Red AC doses and treatment schedules were similar in the present study and the Comet assays performed by Tsuda et al., differences in Comet assay protocols may have led to the ensuing discrepancies of the result in the glandular stomach between the two studies. In particular, two Comet specimens are allowed in the OECD TG 489 guideline, isolated cells and isolated nuclei, which are generally prepared by mesh filtration and homogenizing of minced tissues, respectively. Although no essential differences of results have been demonstrated between isolated cells and isolated nuclei (Tice et al., 2000), Nakajima et al. (2012) suggested that the isolation of nuclei using a Potter-type homogenizer, as in the study by Tsuda et al., leads to significant DNA damage during the Comet preparation, and recommended the use of a Dounce-loose-type homogenizer to prepare intact nuclei for Comet assays. Other protocols for cell lysis and unwinding as well as electrophoresis conditions differed between the present study and that reported by Tsuda et al. In Comet evaluation, moreover, we measured percentages of DNA in tails, Olive tail moments, and tail lengths were determined, and finally judged the result by percentages of DNA in tails as recommended by the OECD TG489 guideline, whereas Tsuda et al. primarily assessed "mitigation of nuclear DNA" according to the lengths of whole Comets and diameters of Comet heads. These technical differences may be the source of differing results.

However, we did not examine colon tissues in the present study, which yielded the strongest DNA damage in the study of Tsuda et al. Some sulphonated aromatic amine metabolites of Allura Red AC, possibly produced from azo-reduction in gastrointestinal tracts, may have genotoxic potential (Jung et al., 1992). Thus, further studies are required to examine the mutagenic properties of these metabolites and may resolve the present controversies. In conclusion, the present data show no evidence of the *in vivo* genotoxic potential of Allura Red AC administered up to maximum doses in mice.