

## 実験技術

# 分析対象の有機化合物の純度は大丈夫ですか？ 定量 NMR による絶対純度測定法の開発

杉本 直樹

**要約：**従来の手法では、有機化合物の絶対純度を簡単に測定することが困難であった。定量核磁気共鳴法（定量 NMR: quantitative NMR (qNMR)）は計量学的に信頼性の高い定量値または純度値を求めることができる強力なツールとして注目を集め始めている。<sup>1</sup>H-NMR は、特に有機化合物の構造決定のための代表的な定性分析法の 1 つであり、これは官能基上の水素の数と信号強度が比例することを利用して、<sup>1</sup>H-NMR スペクトル上に観察される水素の数を示す信号強度は 10% を超えるばらつきがあり、有機化合物の精密な定量分析には不向きであるとされていた。しかし、近年、定性的な NMR 測定条件を全面的に定量用に最適化することで、<sup>1</sup>H-NMR スペクトル上の化合物の水素の信号強度は結合状態に依存せず分子構造が異なっても等モル量であれば等しく観察されることが見出された。この定量的な NMR 現象を利用することによって、qNMR は他の定量分析法に匹敵する不確かさ約 1% 以内の定量精度を実現した。さらに、これまでの定量分析技術の常識を覆し、たった 1 つの純度既知の基準物質を上位標準とするだけで無限の有機化合物の絶対量や絶対純度が国際単位系 (SI) にトレーサブルに求められるようになった。今後、qNMR は多分野の研究に関連する有機化合物の絶対純度決定法として応用がはじまり、得られた分析値や評価値の信頼性を間接的に裏付けるための必須の分析技術となると考えられる。本稿では、有機化合物の純度に関する SI トレーサビリティの重要性、qNMR の原理、市販標準品や試薬の絶対純度測定への応用例などを紹介する。

## 1. SI トレーサビリティの重要性

有機化合物の定量値だけではなく生理活性値などの

あらゆる分析値・評価値の信頼性だけでなく、近年のグローバル化に伴い、得られた分析結果に対する国際的な整合性も大きく問われるようになってきた。分析結果の信頼性・整合性を確保するために、繰り返し精度はもちろんのこと、さらにはバリデーションあるいはクロスチェックの実施が要求される。この際、分析・評価対象の有機化合物の標準品には純度が正確に値付けられていることが前提となっており、実際に、標準品あるいはその代用に用いられた市販試薬製品に表示された純度値や含量値の真否が具体的に問われることは通常まれである。仮に少しでも純度に疑義がある場合は同じロットの製品を用いることで純度の差異に由来する実験誤差を極力小さくし、その研究方法や実験系自体の再現性を確保しているのが現状である。従って、市販試薬や標準品の絶対的な純度や含量値の決定法に関しては、サイレントで大きな問題が残されたままである。

物質量の絶対値は、国際単位系 (International System of Units: SI) にトレーサブルな測定によって得られると定義されている。このような測定法は「一次標準測定法」と呼ばれている。「一次標準測定法」の資格を有する分析法は「一次直接法」と「一次比率法」に分類される。「一次直接法」は、「物質量の基準となる他の化学物質を用いずに、自分自身で目的の化学物質の物質量を測れる方法 (絶対測定法)」であり、電量分析法、重量分析法および凝固点降下法がある。これらの分析法は物質量の絶対値が得られるが、分析できる物質の種類に限られる。一方、「一次比率法」は、「物質量の基準となる別の化学物質を用い、それとの比較において目的の化学物質の物質量を測れる方法」であり、すでに実用化されているものに滴定法および

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国立医薬品食品衛生研究所 生活衛生化学部 (〒158-8501 東京都世田谷区上用賀 1-18-1)

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Author: Naoki Sugimoto



物 B の特定の置換基上の水素のシグナル面積 ( $I_B$ ) についても同様な関係式が当然成り立つ (式 1 の分母)。よって、2 つのシグナルが異なる化合物 (A, B) に由来する場合には個々のシグナル面積と化合物のモル濃度は式 1 の関係式で表すことができる。さらに純度が明らかな化合物を基準物質として用いれば、モル比と溶液の調製値の関係からあらゆる測定対象の有機化合物の純度を決定できる式 2 の関係が成り立つ。qNMR は、式 2 を利用した分析技術である。従って、式 2 からわかるように「物質量の基準となる別の化学物質を用い、それとの比較において目的の化学物質の物質量を測れる方法」であり、前述の「一次標準測定法」のうち「一次比率法」の資格を有する測定法であると言える (図 1 右)。

$$\frac{I_A}{I_B} = \frac{H_A m_A}{H_B m_B} = \frac{H_A W_A / M_A}{H_B W_B / M_B} \quad (\text{式 1})$$

$$P_{\text{sample}} = \frac{I_{\text{sample}} / H_{\text{sample}}}{I_{\text{std}} / H_{\text{std}}} \times \frac{M_{\text{std}} / W_{\text{std}}}{M_{\text{sample}} / W_{\text{sample}}} \times P_{\text{std}} \quad (\text{式 2})$$

$I$  = シグナル面積,  $H$  = 特定基のプロトン数,

$m$  = モル濃度,  $W$  = 重量,  $M$  = 分子量,  $P$  = 純度%,

sample = 測定対象の有機化合物, std = 基準物質。

測定対象の有機化合物の純度値の SI へのトレーサビリティの確保のために、基準物質は測定対象とは別の計量学的に正確に値付けられた上位標準である必要がある。いくつかの有機化合物が上位標準の候補として既に報告されていたが、測定対象の有機化合物のシ

グナルが重ならないことや重溶媒に対する溶解性等、測定対象毎に測定条件を考慮する必要がある点で汎用性が乏しく、これが qNMR の有効利用を妨げる最大の弱点となっていた。そこで、計量学的に正確に純度が値付けられ SI トレーサビリティ源として使用可能である 2-ジメチル-2-シラペンタン-5-スルホン酸- $d_6$  ナトリウム塩 (DSS- $d_6$ ) (水系用) および 1,4-ビス(トリメチルシリル)ベンゼン- $d_4$  (1,4-BTMSB- $d_4$ ) (非水系用) が qNMR 用の基準物質として新たに開発された。DSS- $d_6$  と 1,4-BTMSB- $d_4$  は、テトラメチルシラン (TMS) と同様に 0 ppm 付近にシグナルを示すため、測定対象の有機化合物のシグナルと重なってしまうことや溶解性等をほとんど考慮することも必要としない。さらに、これらを qNMR 用の基準物質として設定することで qNMR スペクトルは通常の構造解析用のスペクトルとしても利用することが可能となり、汎用性と利便性が大幅に改善されている。図 2 に、qNMR による絶対純度測定法の実際の手順を示した。基準物質と分析対象化合物を精密に秤取り混合したものを qNMR 最適条件下で測定し、qNMR スペクトル上に観察される両者のシグナル面積を求め、式 2 に化合物情報等を代入して絶対純度を算出する。得られる測定値は高い再現性を示し、不確かさは概ね 1% 以内であり、1 測定当たりの所要時間は約 10~20 分である。

また、本稿が掲載される時点では、qNMR 測定条件、自動解析ソフトウェアおよび qNMR 基準物質等の配

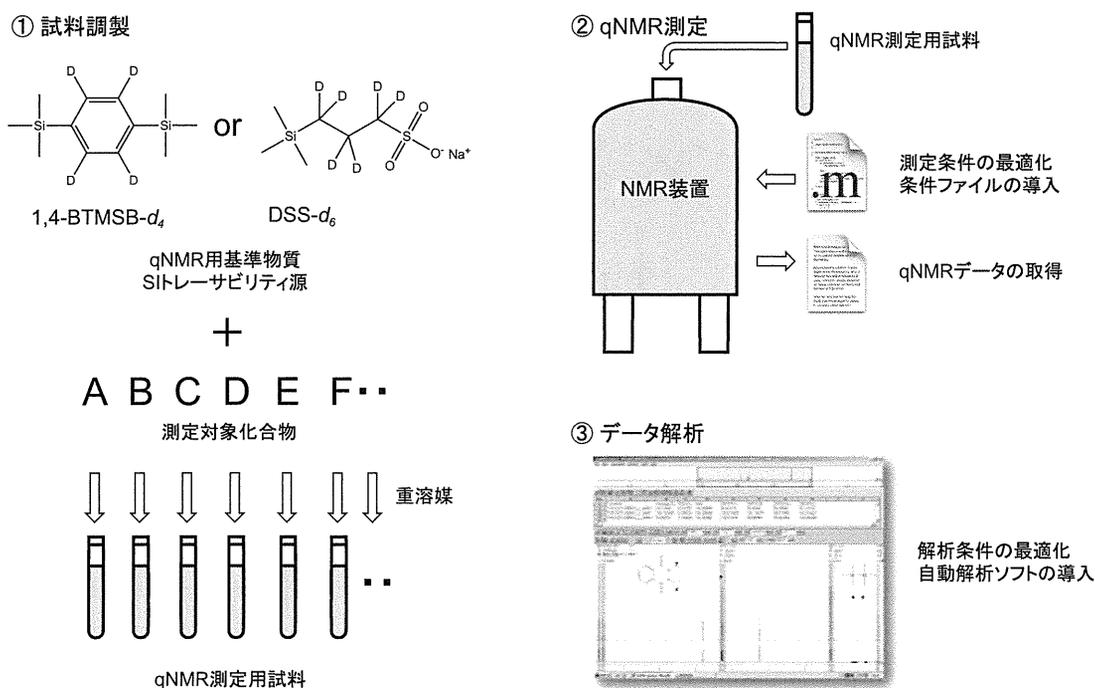


図 2 SI トレーサビリティを確保した qNMR による絶対純度測定法の実際の手順

布が開始され、これまでの「一次標準測定法」とは一線を画した1つの純度既知の基準物質を参照するだけで無限の有機化合物の絶対量や絶対純度がSIにトレーサブルに簡便かつ迅速に求められる、すなわち、「1対多型校正技術」による標準物質供給システムが実用化されていると思われる(図1右)。

### 3. qNMR の応用例(3-6)

有機リン系農薬の1つであるイソキサチオンオキソン(IXO)の市販標準品の絶対純度決定への応用例を示す。冷蔵保存しておいた残留農薬試験用IXO標準品2ロットについて、qNMR測定を行い、IXOの各置換基と基準物質(ここではヘキサメチルジシラン:HMD)のシグナル面積比より絶対純度を求めた。その結果、ロット1とロット2の純度値は大きく異なり、ロット1が75.4%、ロット2が98.5%であり、ロット1については試薬会社の表示値と大きく異なった。ロット1のqNMRスペクトル上にはIXOのシグナル以外に分解物に由来するいくつかのシグナルが観察され、保存中の分解によって絶対純度の低下が引き起こされたと考えられるものであった(図3)。従って、市販標準品として入手したものであっても、保存状態等により、その絶対純度と品質はロット毎に変化することが示唆された。食品残留農薬のポジティブリスト制度の導入により、国内外で流通する農薬が規制の対象となり、食品の安全性と直結する残留農薬試験には高い分析精度が求められている。qNMRはこれらの残留農

薬試験用標準品について絶対純度を付加できるだけでなく、日常的な品質管理に応用可能であり、科学的根拠に基づいた分析値の信頼性の確保にも重要であると言える。

次に、天然有機化合物の市販試薬、標準品および単離精製品の絶対純度測定に応用例を示す。カルミン酸(CA)は天然由来の赤色素で主に着色料に用いられている。試薬会社により純度値が>70%および>95%とラベル表示されたCA市販試薬A、Bを入手し、qNMRにより絶対純度を求めたところ、それぞれ21.3%および78.3%であった。CA市販試薬のラベル表示値が、試薬会社が独自の品質保証の目的でLC法または吸光度法により値付けただけであり、絶対量を示すものではないことが明らかとなった。また、代表的なフラボノイドであるクエルセチンやルチンの市販試薬各社製品について絶対純度を求めたところ、クエルセチン市販試薬製品については86.0%~92.9%(無水物として換算)、ルチン市販試薬製品については71.0%~90.2%(無水物として換算)と試薬製品によって大きく異なることが明らかとなった。これらの結果は、qNMRが天然物化学の分野で最も古くからある問題であった天然有機化合物の純度の問題を、根本的に解決する計量学的に正確な絶対純度測定法あるいは絶対定量法として有望であることを示している。

今後、qNMRは日本薬局方などの公定書や公定法に記載される分析用標準品の絶対純度決定にも応用可能であると考えられる。なぜなら、規格値や基準値など

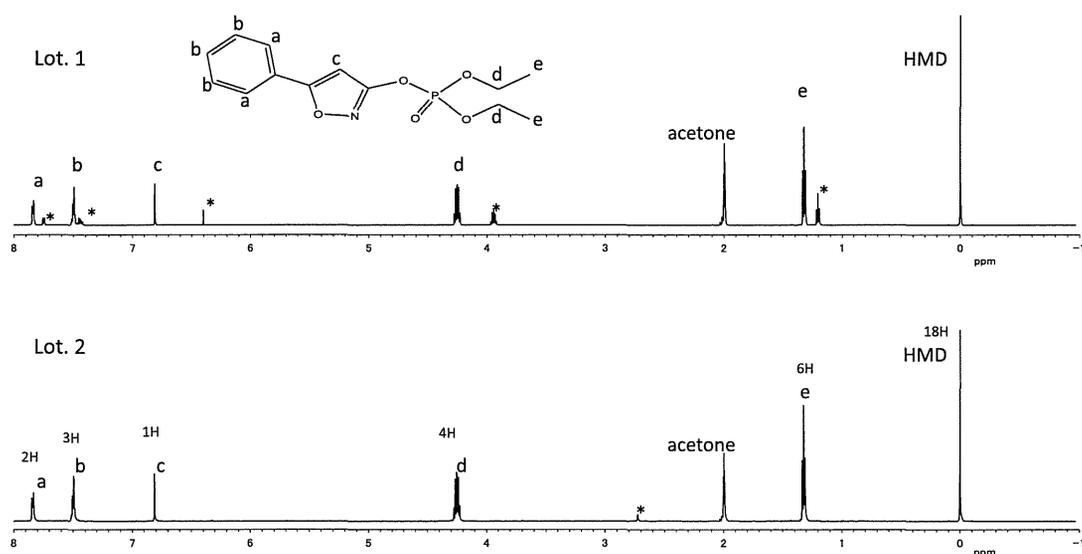


図3 残留農薬試験用イソキサチオンオキソン(IXO)標準品(ロット1,2)のqNMRスペクトル  
qNMR用に測定条件を最適化したJNM-ECA(600MHz)を用いた。重溶媒=アセトン- $d_6$ 、基準物質=ヘキサメチルジシラン(HMD)( $C_6H_{18}Si_2$ )。HMDとIXOのa~eのシグナル強度比より絶対純度を算出した。a~eは各シグナルの帰属。\*は分解物または不純物に由来するシグナル。

の定量精度を確保するためには分析用標品となる試薬や標準品の絶対純度が科学的根拠に基づいて求められているべきであるからである。たとえば、生薬および漢方処方エキスの各条規格などにその応用が有望視されている。生薬および漢方処方エキスの各条規格では、便宜上その時点で市販されている、あるいは市販可能な試薬について規格を試薬・試液の項で定め、その物質を分析用標品として、生薬および漢方処方エキス中の指標成分の定量法（成分含量測定法）と定量規格を規定している。しかし、天然由来の有機化合物は化学合成が困難であり、多くの場合、天然素材より単離精製したものに試薬会社独自の方法により純度の値付けがなされ、市販試薬あるいは標準品として流通している。これらは、原料、抽出方法、単離精製工程の差異により、共存する不純物組成やロット間の絶対純度の変動が化学合成された化合物に比べて大きいと予想され、これが結果として科学的根拠に基づいた生薬類の規格設定のボトルネックになっている。そこでこの問題を解消すべく、生薬に関連する日本薬局方の試薬、市販標準品や研究者が天然素材よりクロマトグラフィーにより単離精製した数種の天然有機化合物についてqNMRによる絶対純度測定を試みたところ、一定の評価ルールの下、これらの絶対純度が規格化できることが明らかとなってきている。

なお、本稿で示した応用例では、qNMRの技術開発を開始した当初、SIトレーサビリティ源となるDSS- $d_6$ と1,4-BTMSB- $d_4$ が開発段階であり、市場に流通していなかったため、qNMRによって得られる定量値のSIへのトレーサビリティは、認証標準物質（certified reference material: CRM）であるフタル酸水素カリウム（PHP）またはジエチルフタレート（DEP）を一次標準（上位標準）として用い、qNMR溶液中のDSS- $d_6$ またはHMDの濃度を校正した後に、これらを二次標準として測定対象化合物のqNMR測定を行う2段階の方式で実現していることをご了承いただきたい。

## おわりに

計量学的に正確に純度が値付けられた標準品（標準物質）の供給・入手が今なお困難であることには変わりはない。分析・評価対象の標準物質が入手できない場合には、市販試薬や単離精製品を代用とすることが定法となっている。しかし、これらの純度の誤差が得られた結果に大きな影響を与えているのは紛れもなく事実である。qNMRは、SIにトレーサブルな1つの上位標準を参照して無限の数の有機化合物の絶対純度あるいは絶対量を正確に算出可能であることから、有機化合物の純度の問題を根本的に解決する方法として注目されている。また、「実用標準物質」の供給体系も根本的に刷新することができる。実際に国内外の試薬会社や標準物質を扱う研究機関において、本稿で述べたqNMRの導入が開始されており、計量学的に正確に値付けられたアミノ酸や農薬の市販標準品の供給体制が整いつつある。また、天然有機化合物、環境汚染物の市販標準品の供給も予定されており、多分野における標準物質の供給が期待されている。今後、SIトレーサビリティの確保は、分析・評価結果の信頼性に関して重要な「鍵」となることは間違いない。qNMRはその「鍵」を迅速かつ簡便に提供することから、分析化学者だけでなく生化学者による応用を強く期待したい。薬物や化合物の純度の正確な評価は、薬理学的生物効果の正確な評価体制の構築にも貢献するであろう。

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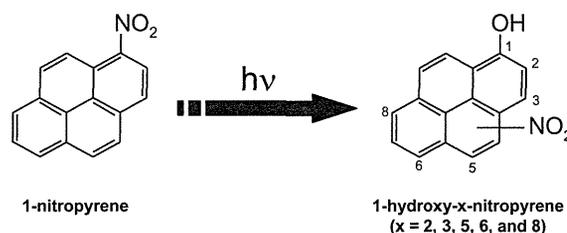
# Atmospheric Formation of Hydroxynitropyrenes from a Photochemical Reaction of Particle-Associated 1-Nitropyrene

Takayuki Kameda,\* Ayuko Akiyama, Akira Toriba, Ning Tang, and Kazuichi Hayakawa

Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan

Supporting Information

**ABSTRACT:** The formation of hydroxynitropyrene (OHNP) via a photochemical reaction of 1-nitropyrene (1-NP) was demonstrated using a UV irradiation system. The photoreaction of 1-NP in methanol gave products that were hydroxy-substituted at position 1 and mononitro-substituted at positions 2, 3, 5, 6, and 8 [1-hydroxy-*x*-nitropyrenes (1-OH-*x*-NPs); *x* = 2, 3, 5, 6, and 8]. 1-OH-2-NP and 1-OH-5-NP have been identified in ambient airborne particles for the first time. On the contrary, these two OHNP isomers were not found in standard reference materials (SRM) 1650b and SRM 1975, which are typical samples of diesel exhaust particles (DEPs). The concentrations of the other OHNP isomers in the DEP samples were much lower than the concentration of 1-NP, which is a representative nitro-derivative polycyclic aromatic hydrocarbon that is emitted directly from combustion sources. On the other hand, significantly higher concentration ratios of  $\Sigma$ OHNP (=1-OH-3-NP + 1-OH-6-NP + 1-OH-8-NP) to 1-NP were observed in ambient airborne particles than in the DEP samples. In ambient airborne particles, the mean  $\Sigma$ OHNP/1-NP concentration ratio of 1.4 was 35 times higher than that in SRM 1650b and 470 times higher than that in SRM 1975. The diurnal concentration of 1-NP, which was observed at a typical residential area in Osaka, Japan, increased early in the morning and late in the evening, suggesting that automotive emissions contributed to the occurrence of 1-NP. The OHNP concentrations also rose in the morning, and variations of OHNP concentrations similar to those of 1-NP were observed during the daytime. However, the concentrations of OHNPs did not increase in the evening rush hour, and were low at night, i.e., in the absence of sunlight. These results support the idea that atmospheric OHNPs are predominantly formed via secondary formation processes; i.e., photochemical reactions of 1-NP are expected to have a significant effect on the occurrence of OHNPs in the atmosphere.



## INTRODUCTION

Nitrated polycyclic aromatic hydrocarbons (NPAHs) are a class of mutagens/carcinogens found in the atmosphere, and some of them exhibit stronger mutagenicity/carcinogenicity than their parent polycyclic aromatic hydrocarbons (PAHs).<sup>1</sup> Some types of NPAHs are formed via gas-phase reactions of semivolatile PAHs. For example, 2-nitropyrene is formed from the gas-phase reaction of pyrene with OH radicals in the presence of NO<sub>2</sub>,<sup>2</sup> and 2-nitrofluoranthene is formed via two pathways, i.e., OH or NO<sub>3</sub> radical-initiated reactions in the gas phase.<sup>2</sup> On the contrary, 1-nitropyrene (1-NP), one of the most abundant NPAHs in the atmosphere, is a representative NPAH formed through combustion of fossil fuels such as diesel fuel.<sup>3</sup> 1-NP taken up by humans and animals is transformed to various metabolites such as hydroxynitropyrenes (OHNPs) in the presence of cytochrome P450 enzymes.<sup>4</sup> Several isomers of OHNP, such as 1-hydroxy-3-nitropyrene (1-OH-3-NP, equivalent to 3-hydroxy-1-nitropyrene), 1-hydroxy-6-nitropyrene (1-OH-6-NP, equivalent to 6-hydroxy-1-nitropyrene), and 1-hydroxy-8-nitropyrene (1-OH-8-NP, equivalent to 8-hydroxy-1-nitropyrene), have also been observed in airborne particles<sup>5</sup> and diesel exhaust particles (DEPs).<sup>3,6,7</sup> Several studies have found that most OHNP isomers have lower mutagenic activity than the parent 1-NP.<sup>7-9</sup> Recently, however, we have found that 1-OH-3-

NP, 1-OH-6-NP, and 1-OH-8-NP act as endocrine disruptors, i.e., they act as estrogenic, antiestrogenic, and antiandrogenic compounds,<sup>10</sup> which may cause dysfunction of human and wildlife endocrine systems, abnormal development of reproductive systems, and immunodeficiencies. In view of the influence of OHNPs on human health, we need to learn more about their environmental concentration levels, sources and behavior.

Numerous photoreaction studies of 1-NP have been performed both in solvents (e.g., methanol,<sup>11-13</sup> benzene,<sup>13,14</sup> acetonitrile,<sup>13,15</sup> dimethyl sulfoxide (DMSO),<sup>16,17</sup> 2-propanol,<sup>13,18</sup> diisooctylphthalate,<sup>19</sup> carbon tetrachloride,<sup>13</sup> 3-methylpentane,<sup>13</sup> toluene,<sup>13</sup> ethanol,<sup>13</sup> hexane,<sup>13</sup> and cyclohexane<sup>13,20</sup>) and on solid substrates (e.g., glass plates,<sup>21</sup> silica,<sup>16</sup> coal fly ash,<sup>22</sup> cellulose,<sup>12</sup> diesel soot particles,<sup>23</sup> and wood smoke particles<sup>23</sup>). The products of these reactions include OHNPs such as 1-hydroxy-2-nitropyrene (1-OH-2-NP),<sup>11</sup> 2-hydroxy-1-nitropyrene (2-OH-1-NP),<sup>15</sup> and 9-hydroxy-1-nitropyrene (9-OH-1-NP)<sup>14</sup> as well as 1-hydroxypyrene (OHPy),<sup>11</sup> pyrenediones,<sup>17,21</sup> and pyrene.<sup>11</sup> So far, only one of these OHNPs, 9-OH-1-NP, has been

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detected in airborne particles.<sup>14</sup> Although 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP have been observed in ambient samples,<sup>5</sup> there is not yet any evidence that they are formed from the photoreaction of 1-NP. In this study, therefore, we examined the formation of OHNPs including 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP from photochemical reactions of 1-NP by laboratory experiments in order to clarify the occurrence of atmospheric OHNPs. The photochemistry of chemicals associated with combustion particles is believed to occur in an organic liquidlike layer surrounding an elemental carbon core.<sup>24</sup> However, the organic phase of ambient aerosols is very complex, and the composition of it remains very uncertain.<sup>19</sup> Therefore, we selected methanol as a simple chemical model of aerosol surface, although it can not be regarded as a “complete” aerosol model system. Furthermore, some particle-associated OHNP isomers that were found in the laboratory photoreaction products were also detected in the atmosphere and diesel engine exhaust.

## EXPERIMENTAL SECTION

**Experimental Setup for Photoreaction of 1-NP.** Photoreaction of 1-NP was performed in a Pyrex sleeve (1 cm in thickness) which surrounded an annular Pyrex vessel (6.6 cm i.d. × 60 cm length) (Supporting Information (SI) Figure S1). The external sleeve has a port for sampling the photoreaction products and the precursor 1-NP. The radiation equipment has six black-light lamps (20 W, Toshiba, FL20S-BLB) and a cooling device for isothermal reaction conditions at  $299 \pm 2$  K. The  $1 \times 10^{-5}$  mol L<sup>-1</sup> of 1-NP in methanol in the external sleeve was irradiated by the black light lamps placed around the reaction vessel under the presence of air. The total incident photon flux reaching to the surface of the sleeve measured with a Hatchard-Parker actinometer using potassium ferrioxalate photoreduction was  $5.2 \times 10^{16}$  photons cm<sup>-2</sup> s<sup>-1</sup>. Products collected after 2 h of the reaction were identified by liquid chromatographic–tandem mass spectrometric (LC/MS/MS) analysis by comparing with the retention times and fragmentation patterns of authentic standards after acid–base and preparative high-performance liquid chromatograph (HPLC) fractionations. <sup>1</sup>H NMR, gas chromatographic–mass spectrometric (GC/MS), and exact mass analyses were also performed for identification of the photoreaction product of which an authentic standard was not available. Quantification of the reaction products was performed by HPLC equipped with a chemiluminescence detector (HPLC/CL) without any sample purification in order to avoid the loss of the products through the purification steps. The irradiation experiments of 1-NP in different solvents [ethanol, acetonitrile, and acetonitrile/water (3/1, v/v)] were also performed by the same procedure to evaluate the solvent effects on the isomer distribution of the photoreaction products.

**Airborne Particle Collection.** Airborne particles were collected at the rooftop level of a three-story building approximately 10 m above ground level at Osaka Prefecture University, Sakai, Osaka, Japan. This sampling site is located in a slightly polluted residential area. The sampling campaign was performed using a high-volume air sampler (Kimoto Electric, model 120) on quartz fiber filters (QFF; Advantec MFS, QR100), at a flow rate of 1500 L min<sup>-1</sup>, during May 12 and 14, 2003 with a regular collection time of 3 h per filter; thus, 20 sample filters were obtained. The airborne particle samples were stored at 253 K until subjected to analysis.

**Extraction of Soluble Organic Fractions from DEP and Airborne Particulate Samples.** Standard reference materials

(SRM) 1650b (diesel particulate matter) and SRM 1975 (diesel particulate extracts) was obtained from U.S. National Institute of Standards and Technology (NIST). Soluble organic fractions (SOFs) were extracted from SRM 1650b using Soxhlet extraction technique according to the previous literature.<sup>3</sup> SRM 1975 was dissolved in methanol at an appropriate concentration.<sup>25</sup> The airborne particulate samples were prepared according to the previous report<sup>26</sup> (details are presented in SI). An aliquot of each of the sample solutions without any purification was subjected to the quantification by HPLC/CL. Correction for chemical loss of OHNPs on a QFF by ozone during the ambient sample collection was performed for the quantification of the particle-associated OHNPs (SI Table S1) according to the previous report.<sup>26</sup>

**Sample Fractionation.** In order to identify the OHNPs in airborne particles and in 1-NP photoreaction products by MS and/or <sup>1</sup>H NMR analyses, sample fractionation was performed. Details are presented in SI.

**Analytical Instrumentation.** LC/MS/MS analysis was performed as previously described<sup>4</sup> using the Agilent 1100 series LC system (Agilent Technologies) with an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems) equipped with an electrospray ionization (ESI) interface and operated in a negative ion mode. The mass spectrometer was operated under multiple reaction monitoring (MRM) mode, and the monitored precursor (Q1) and product (Q3) ions were *m/z* 262 and 232, respectively. The structures of 1-NP photoreaction products were elucidated using the enhanced product ion (EPI) scan mode (details are presented in SI).

An HPLC system with column-switching and chemiluminescence detection was employed for OHNPs and 1-NP quantification as reported previously<sup>26</sup> (details are described in SI).

Details of <sup>1</sup>H NMR, GC/MS, and exact mass analyses performed in this study are presented in SI.

**Chemicals.** 1-OH-3-NP, 1-OH-6-NP, 1-OH-8-NP, and their deuterates were synthesized according to the previously reported procedure.<sup>4</sup> 1-OH-2-NP was synthesized by nitration of OHPy by 4-nitro-4-methyl-2,3,5,6-tetrabromo-2,5-cyclohexadien-1-one (NCHD) in diethyl ether at room temperature for 2 h according to the literature.<sup>27,28</sup> Each OHNP isomer was purified by preparative normal phase HPLC (SPELCO, Supelcosil PLC-SI, 21.2 mm i.d. × 250 mm, eluted with CH<sub>2</sub>Cl<sub>2</sub> containing 0.5 mM CH<sub>3</sub>COOH at 10 mL/min). To identify the synthetic compounds, their EI-MS and <sup>1</sup>H NMR spectra were compared with literature data.<sup>8,11,28–30</sup> NCHD was purchased from Tokyo Chemical Industry Co., Ltd. 1-NP and OHPy were obtained from Sigma-Aldrich Co. Deuterated 1-NP (1-NP-*d*<sub>9</sub>) was obtained from C/D/N Isotopes Inc. All solvents and other chemicals used were HPLC or analytical grades from Wako Pure Chemical Ind., Ltd.

**Measurement of CO and O<sub>3</sub>.** Concentration of CO in the atmosphere was monitored throughout airborne particulate sampling using an NDIR CO analyzer (Thermo Electron, MODEL 48). Concentration of atmospheric O<sub>3</sub> was obtained by public environment monitoring stations in Sakai, Osaka, Japan.

**Computational Methods.** Optimization of the geometry and calculation of atomic spin densities for the pyrenyloxy radical were performed by density functional theory (DFT) method at B3LYP/6-31+G(d) level of theory using the Gaussian 03W programs.

## RESULTS

**Photoreaction of 1-NP.** Figure S3 in SI shows a profile of the preparative HPLC with UV absorption for the products from

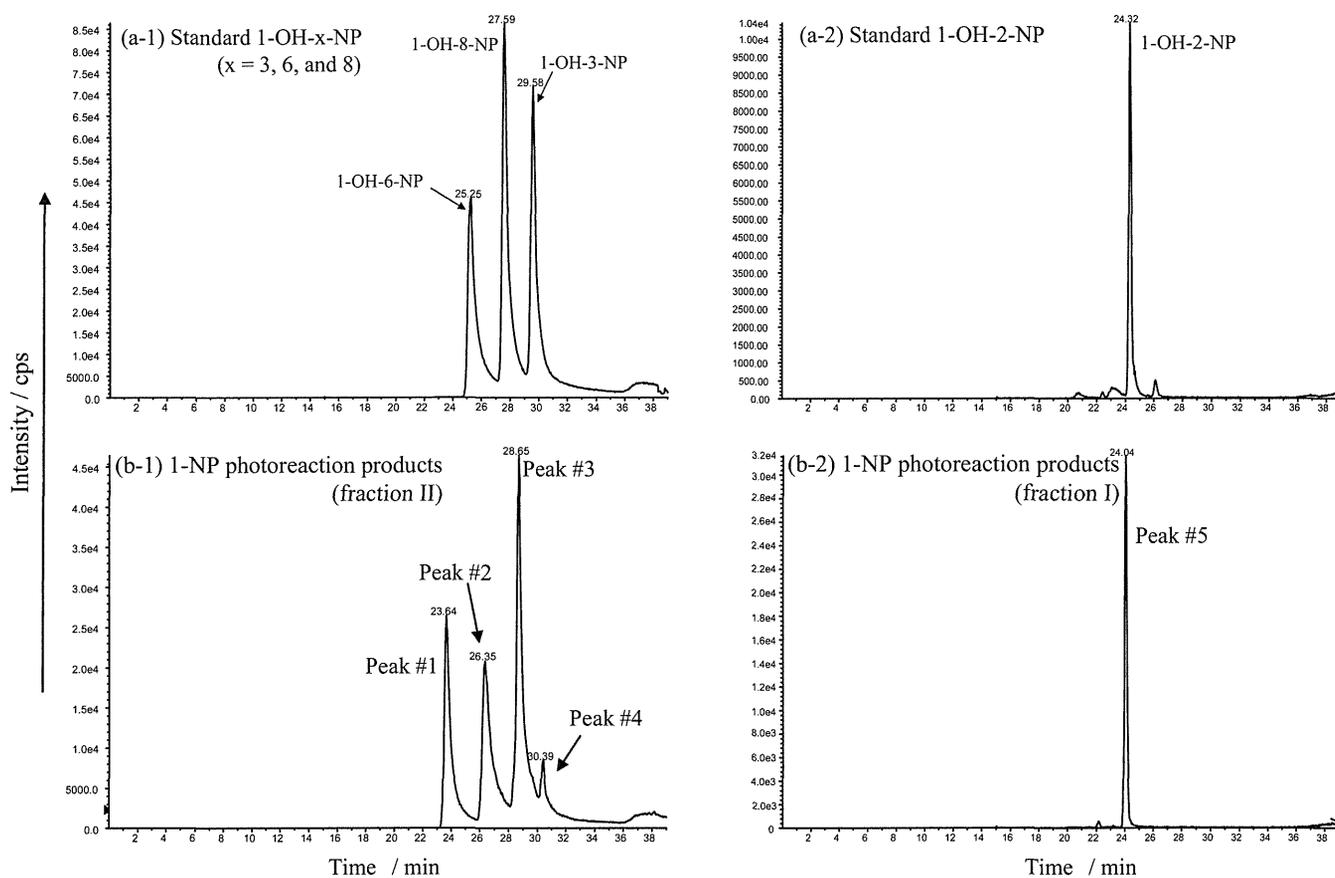
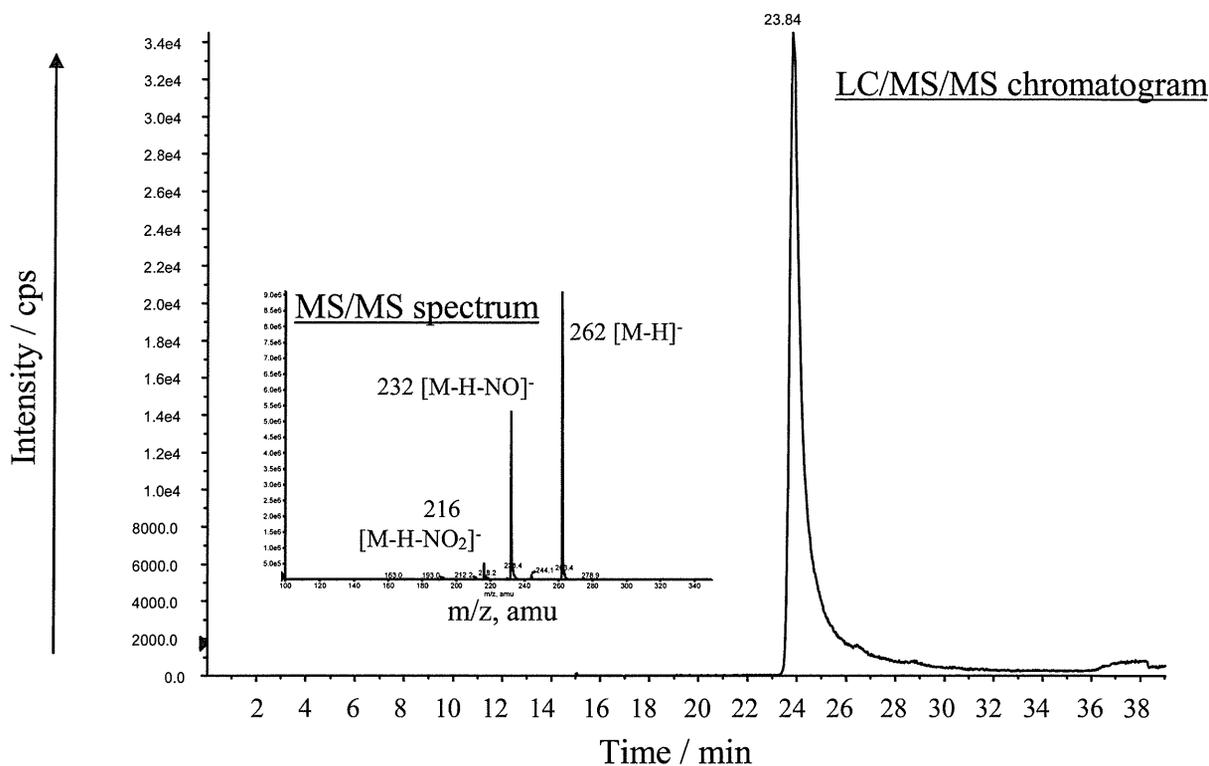


Figure 1. LC/MS/MS chromatograms for (a) standard solutions and (b) photoreaction products of 1-NP;  $m/z$  262 $\rightarrow$ 232.

photoreactions of 1-NP in methanol. Six chromatographic peaks were observed in the chromatogram as symbolized by A, B, C, D, E, and F. The retention times of peaks A, B, D, E, and F were the same as those of authentic 1-OH-2-NP, OHPy (1-hydroxypyrene), 1-OH-6-NP, 1-OH-8-NP, and 1-OH-3-NP, respectively. When analyzed by LC/MS/MS, fraction I containing peak A yielded one peak (Figure 1b-2), and fraction II containing peaks C, D, E, and F yielded four peaks (Figure 1b-1). By comparing the retention times and the MS/MS spectra of these peaks with those of the authentic standards, four known OHNPs, 1-OH-2-NP, 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP, were identified. For these compounds, the molecular-related ion  $m/z$  262 ( $[M - H]^-$ ) together with the characteristic fragment ions  $m/z$  232 ( $[M - H - NO]^-$ ) and 216 ( $[M - H - NO_2]^-$ ) were detected in an EPI full scan analysis (SI Figure S4). 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP were found in 1-NP photoreaction products for the first time. The unknown compound that was eluted at 23.6 min in the LC/MS/MS chromatogram (peak 1 in Figure 1b-1) was also observed in the LC/MS/MS analysis of fraction III obtained by the HPLC fractionation (Figure 2), which corresponds to the peak C in SI Figure S3. This compound also gave a characteristic MS/MS spectrum with a molecular-related ion  $m/z$  262 and fragment ions  $m/z$  232 and  $m/z$  216 in EPI mode (Figure 2). The similarity between the fragmentation patterns of the unknown compound and known OHNPs indicates that the unknown compound is an isomer of OHNP. The structure of the unknown compound obtained by the preparative scale photoreaction was then determined by analysis of its  $^1H$  NMR spectrum. On the basis of chemical shifts and coupling

patterns (SI Figure S5), the unknown compound contained in the fraction III was identified as 1-hydroxy-5-nitropyrene (1-OH-5-NP).  $^1H$  NMR (acetone- $d_6$ ,  $\delta$  in ppm,  $J$  in Hz): 7.80 (d, 1H, H-2,  $J = 8.5$ ), 8.17 (d, 1H, H-9,  $J = 9.0$ ), 8.18 (t, 1H, H-7,  $J = 7.9$ ), 8.35 (d, 1H, H-8,  $J = 7.6$ ), 8.46 (d, 1H, H-3,  $J = 8.5$ ), 8.49 (d, 1H, H-10,  $J = 9.0$ ), 8.83 (dd, 1H, H-6,  $J = 8.1, 0.8$ ), 9.00 (s, 1H, H-4). The results of MS and UV spectrum analyses are as follows: EI-MS  $m/z$  (rel. int. %): 263 ( $[M]^+$ , 80), 233 ( $[M - NO]^+$ , 100), 217 ( $[M - NO_2]^+$ , 53), 205 ( $[M - CNO_2]^+$ , 38), 189 ( $[M - NO_2 - CO]^+$ , 76), 176 (53), 94 (53), 88 (80). Exact mass calculated for  $C_{16}H_9NO_3$ :  $m/z$  263.0582. Found:  $m/z$  263.0590 ( $\Delta = +2.7$  ppm). UV (methanol)  $\lambda_{max}$  (nm, rel.  $\epsilon$  %): 385 (28), 365 (15), 330 (59), 270 (100). HPLC purity of 1-OH-5-NP obtained by the preparative HPLC fractionation of the photoreaction products was determined at 254 nm to be  $\geq 92\%$ . Standard solution of 1-OH-5-NP in this study was prepared from known amount of the purified 1-OH-5-NP, and was used to quantify 1-OH-5-NP in the photoreaction products and airborne particles. Two kinds of hydroxylated 1-NP, i.e., 2-OH-1-NP and 9-OH-1-NP, were previously reported as 1-NP photoreaction products as well as 1-OH-2-NP.<sup>14,15</sup> However, the EI-MS and/or UV absorption spectra for the compound eluting in fraction III were quite different from those for 2-OH-1-NP and 9-OH-1-NP in the previous reports.<sup>14,15</sup> Although 1-OH-5-NP, which is equivalent to 4-nitro-8-hydroxypyrene, has been reported to be a metabolite of 4-nitropyrene,<sup>31</sup> this study is the first to show that it is also a photoreaction product of 1-NP.

**OHNPs in DEPs and Ambient Airborne Particles.** Table 1 shows the concentrations of OHNPs and 1-NP in SRM 1650b,



**Figure 2.** LC/MS/MS chromatogram ( $m/z$  262 $\rightarrow$ 232) and MS/MS spectrum for fraction III of the 1-NP photoreaction product obtained by the HPLC fractionation.

**Table 1.** Concentrations of Hydroxynitropyrenes and 1-Nitropyrene and Concentration Ratio of Hydroxynitropyrenes to 1-Nitropyrene in Airborne Particles, SRM 1975, and SRM1650b<sup>a</sup>

	1-OH-2-NP	1-OH-3-NP	1-OH-5-NP	1-OH-6-NP	1-OH-8-NP	1-NP	$\Sigma$ OHNP/1-NP <sup>g</sup>
airborne particles <sup>b</sup>	3.4 <sup>d</sup>	0.9 <sup>d</sup>	4.1	16.3	15.7	23.7	1.4
SRM1975 <sup>c</sup>	n.d.	0.011	n.d.	0.030	0.019	18 <sup>e</sup>	0.003
SRM1650b <sup>c</sup>	n.d.	0.09	n.d.	0.32	0.25	15 <sup>f</sup>	0.04

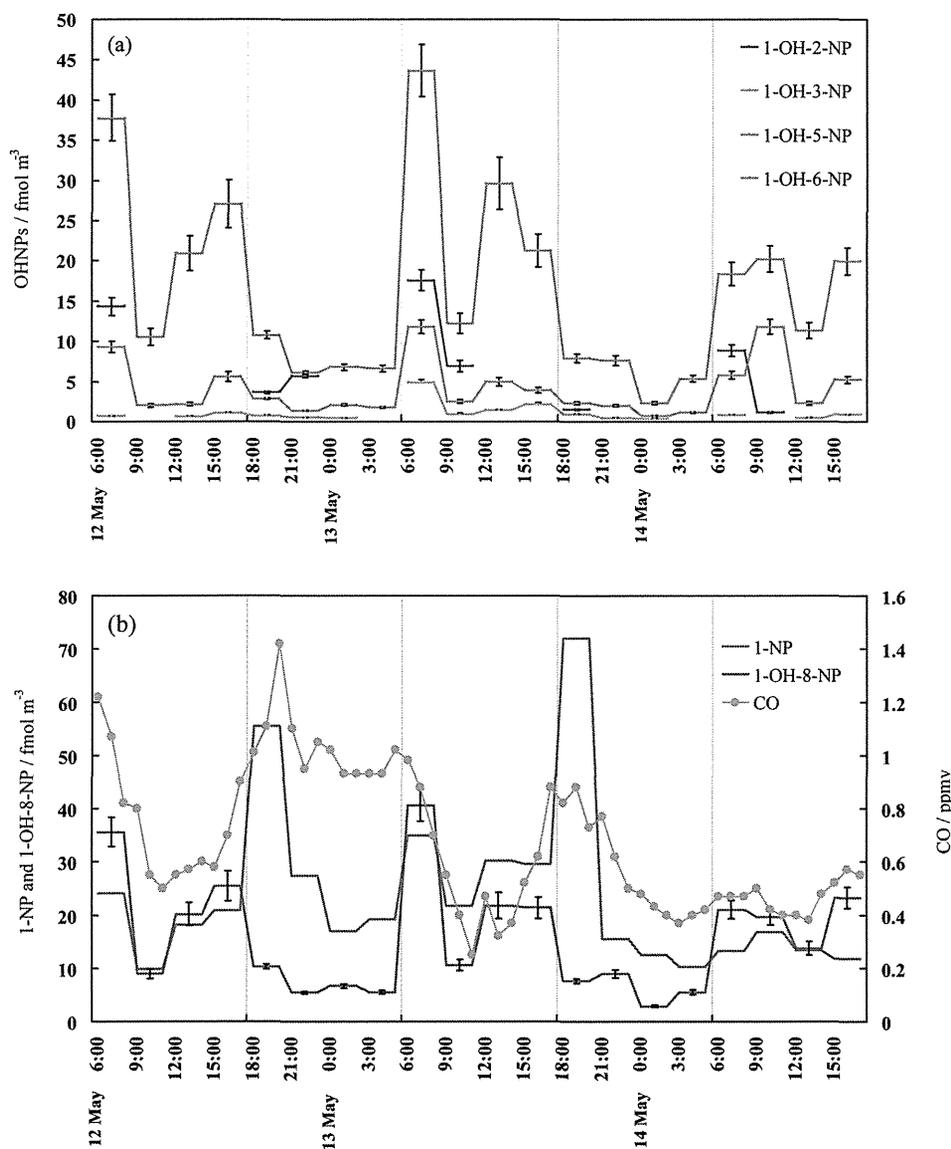
<sup>a</sup> Abbreviations are as follows: 1-OH-*x*-NP, 1-hydroxy-*x*-nitropyrene ( $x = 2, 3, 5, 6,$  and  $8$ ); 1-NP, 1-nitropyrene; n.d., not detected. Detection limits of HPLC analysis employed for 1-OH-2-NP and 1-OH-5-NP were 17 fmol and 0.3 fmol, respectively ( $S/N = 2$ ). <sup>b</sup> Mean values of 20 ambient samples collected during May 12–14, 2003, at Sakai, Osaka, Japan (see text and Table S2 for detail). Given in units of fmol m<sup>-3</sup>. <sup>c</sup> Given in units of mg kg<sup>-1</sup>. <sup>d</sup> Calculated using 0.5 of the minimum detectable values as concentrations lower than the quantification limits. See Table S2 for detail. <sup>e</sup> 112% of the value certified by NIST ( $16.4 \pm 0.2$  mg kg<sup>-1</sup>). <sup>f</sup> 81% of the value certified by NIST ( $18.2 \pm 0.2$  mg kg<sup>-1</sup>). <sup>g</sup>  $\Sigma$ OHNP = 1-OH-3-NP + 1-OH-6-NP + 1-OH-8-NP.

SRM 1975, and airborne particles collected in May, 2003, at a residential area in Osaka, which were determined by the HPLC/CL method. The DEP samples contained 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP but not 1-OH-2-NP or 1-OH-5-NP (SI Figure S6). Manabe et al. and Schuetzle also found 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP in DEPs at concentrations of 10–100  $\mu$ g g<sup>-1</sup> of diesel particulate extract.<sup>3,7</sup> In the airborne particulate samples, on the other hand, 1-OH-2-NP and 1-OH-5-NP were detected for the first time, as well as 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP (SI Figure S7). The mean concentrations of airborne particle-bound 1-OH-2-NP, 1-OH-3-NP, 1-OH-5-NP, 1-OH-6-NP, and 1-OH-8-NP were 3.4, 0.9, 4.1, 16.3, and 15.7 fmol m<sup>-3</sup>, respectively. The concentrations of 1-NP associated with airborne particles (9.8–72.0 fmol m<sup>-3</sup>) were comparable to those reported elsewhere in Japan.<sup>32,33</sup> The OHNPs in airborne particles were also identified, but not quantified, by LC/MS/MS (SI Figure S8). The molecular-related ion,  $m/z$  263 ([M – H]<sup>-</sup>), together with the characteristic fragment ions,  $m/z$

232 ([M – H – NO]<sup>-</sup>) and 216 ([M – H – NO<sub>2</sub>]<sup>-</sup>) were observed, and the fragmentation pattern for each peak obtained from the airborne particulate sample was consistent with that for the authentic OHNP (SI Figure S9).

## DISCUSSION

The concentrations of 1-NP in SRM 1650b and SRM 1975 determined by the HPLC/CL method (Table 1) in this study, 15 and 18 mg kg<sup>-1</sup>, were 81% and 112% of the values certified by NIST, respectively.<sup>34,35</sup> The concentrations of DEP-associated 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP, 0.09–0.32 mg kg<sup>-1</sup> for SRM 1650b and 0.01–0.03 mg kg<sup>-1</sup> for SRM 1975, were much lower than that of 1-NP. The concentration ratios of  $\Sigma$ OHNPs (= 1-OH-3-NP + 1-OH-6-NP + 1-OH-8-NP) to 1-NP in SRM 1650b and SRM 1975 were 0.04 and 0.003, respectively, while the mean concentration ratio of  $\Sigma$ OHNPs to 1-NP associated with airborne particles was 1.4; i.e., the value



**Figure 3.** Diurnal concentrations of (a) OHNPs and (b) 1-NP and CO together with 1-OH-8-NP during May 12–14, 2003, at Sakai, Osaka. The concentration of each OHNP was corrected based on its degradation rate during high volume air sampling. Errors shown were calculated from one standard deviation derived from the correction method employed in this study. See ref 26 for details.

of  $\Sigma$ OHNPs/1-NP in ambient airborne particles was higher than that in DEPs by factors of about 35 and 470. The low  $\Sigma$ OHNPs/1-NP ratio in DEPs estimated as 0.04–0.2 by Schuetzle favorably compares with our results.<sup>3</sup> The difference in the ratio between in DEPs and in airborne particles implies that some OHNPs are formed in the atmosphere. Little is known about the concentrations of OHNPs in airborne particles. Ambient airborne concentrations of hydroxynitro-PAH isomers (MW 263, mainly OHNPs and hydroxynitrofluoranthenes) reported to be about 0.01 ng m<sup>-3</sup> (40 fmol m<sup>-3</sup>) by Nishioka et al. were well consistent with our results.<sup>36</sup> Gibson et al. also quantitatively analyzed airborne particle-bound OHNPs, although they did not determine each OHNP isomer independently.<sup>5</sup> At sites, in Delaware and Bermuda, concentrations of OHNPs, as a mixture of 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP, were also found to be higher than that of 1-NP; the ratio of  $\Sigma$ OHNPs/1-NP in the airborne particles ranged from 1.7 to 21, suggesting secondary formation of OHNPs in the atmosphere, although they did not

confirm the reaction pathway.<sup>5</sup> Our inability to detect either 1-OH-2-NP or 1-OH-5-NP in DEP samples also strongly supports the atmospheric secondary formation of OHNPs, because these OHNP isomers were found in the airborne particles.

Figure 3 and Table S2 in SI show diurnal concentrations of OHNPs with the standard errors in 3-h averaged samples of the airborne particles obtained in this study between May 12 and 14, 2003. The diurnal variations in concentrations of particle-associated OHNP isomers during the sample collection were similar. This suggests that a dominant formation process of these OHNPs is identical, and that the OHNPs have very similar chemical removal rates from the atmosphere. The diurnal changes of 1-NP and 1-OH-8-NP in airborne particles collected between May 12 and 14 are shown in Figure 3b with the concentration of CO, which was measured during the same period of time. The CO concentration was in the range 0.3–1.4 ppmv. The diurnal variations of the concentrations of CO and 1-NP, both of which are primarily emitted by combustion

processes, particularly the combustion of automotive fuels, were similar; i.e., the concentrations increased early in the morning and late in the evening. The OHNP concentrations were also elevated in the morning, and variations of OHNP concentrations similar to that of 1-NP were observed during the daytime (6:00–18:00). On the other hand, the concentrations of OHNP isomers did not increase in the evening rush hour, and were low at night (18:00–6:00), i.e., in the absence of sunlight. The observed diurnal variability of OHNPs clearly indicates that OHNPs originate from not only vehicle emissions but also secondary formation in the atmosphere, probably via the photoreaction of 1-NP.

Van den Braken-van Leersum et al. proposed that the first step of the 1-NP photoreaction in methanol is the formation of a nitrite intermediate via both intramolecular nitro–nitrite rearrangement and C–N bond dissociation-recombination mechanisms, and that the second step is generation of nitrogen oxide (NO) and pyrenyloxy radicals.<sup>11</sup> The NO radicals are expected to recombine with the pyrenyloxy radicals at various carbon positions followed by the formation of OHNPs, although only 1-OH-2-NP was found in their study. In our study, four other OHNP isomers were first identified as 1-NP photoreaction products together with 1-OH-2-NP. These five isomers were also found in airborne particles. Thus, the mechanism of the 1-NP photoreaction proposed by van den Braken-van Leersum et al.<sup>11</sup> may also be responsible for the formation of atmospheric OHNPs. According to their study, the coupling between NO and pyrenyloxy radicals is predicted to occur at the carbon of the cleaved fragment with high spin density. The DFT-calculated spin densities of pyrenyloxy radical were high at carbon positions 2, 5, 6, 8, and 9, but quite low at position 3 (SI Figure S10). Pohlers et al. obtained similar spin densities for the pyrenyloxy radical by a calculation at the AM1-RHF level of theory.<sup>37</sup> These calculations are consistent with the preferential production of OHNP isomers nitrated at positions 2, 5, 6, and 8 over position 3, although 1-OH-9-NP was not observed in our photoreaction experiment or in our ambient samples. The isomer distribution of OHNPs in airborne particles was slightly different from that in the photoreaction experiment products in the solvent; the photoreaction in methanol was highly selective for the formation of 1-OH-2-NP (SI Table S3). The photodecomposition of NPAH in a solvent or on a solid surface strongly depends on several factors, such as the type of solvent, the physical and chemical nature of the substrates, and the presence of other chemicals.<sup>13,20,23</sup> Figure S11 in SI shows the distributions of OHNP isomers obtained from the photoreaction of 1-NP in various solvents as in the case of methanol. The highest relative yield of 1-OH-2-NP was obtained in methanol (44%), followed by ethanol (30%), acetonitrile/water (3/1, v/v) (14%), acetonitrile (9%). The preferential formation of 1-OH-2-NP observed in the photoreaction in hydroxylic and viscous solvents such as alcohol may be attributable to the cage effect; the solvent cage surrounding the reactant is expected to inhibit the migration of the dissociated NO radical, resulting in preferential recombination of NO with the neighboring carbon atom of the pyrenyloxy radical. Another possible formation pathway of OHNPs in ambient air is a reaction of OHPy on the particles, which can be produced from photoreactions of 1-NP or pyrene,<sup>11,38</sup> with gaseous nitrating species such as NO<sub>2</sub>, HNO<sub>3</sub>, and N<sub>2</sub>O<sub>5</sub>. Several researchers reported the reactions of particle-associated PAHs such as pyrene and fluoranthene with the gaseous nitrating species.<sup>39–41</sup> The NPAH isomers formed from the heterogeneous reactions are the same as those formed from electrophilic

nitration reactions involving NO<sub>2</sub><sup>+</sup> ions. The addition of an activating group such as a hydroxyl to the PAH species would be expected to greatly increase the reactivity of the ring toward further reaction, including nitration.<sup>6</sup> Electrophilic nitration of OHPy by NO<sub>2</sub><sup>+</sup> preferentially leads to 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP as in pyrene.<sup>11</sup> Thus, this reaction pathway could partly participate in the formation of 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP in ambient air, although the formations of 1-OH-2-NP and 1-OH-5-NP cannot be explained by this process. Formations of 1-OH-6-NP and 1-OH-8-NP from photoreactions of dinitropyrenes (DNPs) have also been reported,<sup>16,42</sup> but DNP concentrations in ambient air seem too low to contribute to the atmospheric formation of OHNPs; the concentrations of DNPs are 2 orders of magnitude lower than that of 1-NP.<sup>43</sup> Several researchers reported that 1-hydroxy-2-nitronaphthalene and 2-hydroxy-1-nitronaphthalene were produced from the gas-phase reactions of naphthalene initiated by OH or NO<sub>3</sub> radicals in the presence of NO<sub>2</sub>.<sup>44,45</sup> This raises the possibility that atmospheric OHNPs are formed partly by the radical-initiated reactions of pyrene in the gas-phase. OHPy is an expected hydroxypyrene isomer formed from the OH radical-initiated chemistry of gaseous pyrene.<sup>2,44</sup> As described above, OHPy is expected to be reactive toward both OH and NO<sub>3</sub> radicals. Both the OH and NO<sub>3</sub> radical-initiated reactions of OHPy in the gas phase could lead to OHNP formation by analogy with the similar reactions of phenol and cresols,<sup>46</sup> although vapor pressure of OHPy is significantly lower than those of the other species.<sup>47</sup> In order to understand the factors affecting the formation and behavior of atmospheric OHNPs, detailed kinetic experiments and further observation of ambient OHNPs are required.

## ■ ASSOCIATED CONTENT

**S** Supporting Information. Figures S1–S11 and Tables S1–S3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Phone: +81-76-234-4458; fax: +81-76-234-4456; e-mail: [kameda@p.kanazawa-u.ac.jp](mailto:kameda@p.kanazawa-u.ac.jp).

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# Estrogenic/Antiestrogenic Activities of Quinoid Polycyclic Aromatic Hydrocarbons

Kazuichi Hayakawa,<sup>\*,a</sup> Kanae Bekki,<sup>a</sup> Morio Yoshita,<sup>a</sup> Chihiro Tachikawa,<sup>a</sup> Takayuki Kameda,<sup>a</sup> Ning Tang,<sup>a</sup> Akira Toriba,<sup>a</sup> and Shinzo Hosoi<sup>b</sup>

<sup>a</sup>Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920–1192, Japan and

<sup>b</sup>The Research Center for Pharmacy Education, Kyoto Pharmaceutical University, Misasagi-Nakauchicho 5, Yamashinaku, Kyoto 607–8414, Japan

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Estrogenic and antiestrogenic activities of 19 quinoid polycyclic aromatic hydrocarbons (PAHQs) and 9 ketone PAHs were evaluated by the yeast two-hybrid assay using yeast cells expressing estrogen receptor- $\alpha$  (ER $\alpha$ ). Binding affinity of PAHQs to ER $\alpha$  was assayed by the polarized fluorescence method using Fluormone<sup>TM</sup> ES2. Ten PAHQs having 3–5 rings showed antiestrogenic activities. The most strongly antiestrogenic PAHQs were 1,4-chrysenequinone and 5,6-chrysenequinone. On the other hand, benzo[*a*]pyrene-3,6-quinone showed the strongest estrogenic activity. However, the other compounds tested did not show so strong estrogenic/antiestrogenic activities. Binding affinity to ER was required but not sufficient for estrogenic/antiestrogenic activities of PAHQs. The length-to-breadth ratios of the rectangular planes surrounding the ring molecules and the distances between the oxygen atom of the carbonyl group and farthest hydrogen atom of estrogenic/antiestrogenic PAHQs were in narrow ranges, suggesting a structure-activity relationship. As interactions between active PAHQ and ER, hydrogen bonding between carbonyl groups and amino acid residues and van der Waals forces were considered.

**Key words**— polycyclic aromatic hydrocarbon, quinone, antiestrogenic activity, yeast two-hybrid, structure-activity relationship

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. In recent years there has been increasing interest in endocrine disruptors which may cause dysfunction of human and wildlife endocrine systems leading to cancers, reproductive system abnormalities and immune system deficiencies.<sup>1,2)</sup> Several screening tests have been developed to evaluate the endocrine-disrupting activities of chemicals. As for PAHs, antiestrogenic activity was observed in a yeast assay system<sup>3)</sup> and estrogenic activity was found in MCF-7 cells.<sup>4)</sup> The estrogenic activities of 517 chemicals were evaluated by using a yeast two-hybrid assay system based on the ligand-dependent interaction of estrogen re-

ceptor (ER) and its co-activators.<sup>5)</sup>

Several studies have evaluated the estrogenic and antiestrogenic activities of hydroxy PAHs (OHPAHs) such as hydroxybenzo[*a*]pyrene (OHBaP).<sup>4,6,7)</sup> Several other OHPAHs and *n*-propyl *p*-hydroxybenzoate were identified as estrogenic compounds in cigarette smoke condensate.<sup>8)</sup> We previously evaluated estrogenic/antiestrogenic activities of 14 PAHs and 63 OHPAHs having 2–6 rings by yeast two-hybrid assay and found that PAHs did not show any estrogenic/antiestrogenic activity, but several OHPAHs having 3–5 rings showed activity. Especially, OHPAHs having 4 rings such as 3-, 4- and 10-hydroxybenzo[*a*]anthracenes (3-, 4- and 10-OHBaAs) and 2-hydroxychrysene (2-OHCh) showed strong estrogenic activity. Several other OHPAHs having 4 rings such as 2- and 3-hydroxybenzo[*c*]phenanthrenes (2- and 3-OHBcPh), 2-OHBaA and 3-OHCh showed strong antiestrogenic activity. Both length-to-breadth (L/B) ratios of the

\*To whom correspondence should be addressed: Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920–1192, Japan. Tel.: 076-234-4413; Fax: 076-234-4456; E-mail: hayakawa@p.kanazawa-u.ac.jp

rectangular van der Waals planes surrounding the ring molecules and distances between the oxygen atom of the phenol group and farthest hydrogen atom (O-H distance) of the estrogenic OHPAHs were in narrower ranges than those of antiestrogenic OHPAHs. The similarity of the values of OHPAHs to those of estradiol ( $E_2$ ) and diethylstilbestrol (DES) suggested that OHPAHs interact with the ER through hydrogen bonding between the phenol groups of OHPAH and amino acid residues of ER and van der Waals fitting between benzene rings.<sup>9)</sup>

PAHs are oxidized to OHPAHs, PAH quinones (PAHQs) and ketones *etc.* in the presence of cytochrome P450 enzymes (CYPs), epoxide hydrase and/or aldo-keto reductase in human and animals.<sup>10)</sup> These metabolites are also secondarily formed from mother PAHs in the atmosphere.<sup>11)</sup> Both biological and chemical formations of OHPAHs or PAHQs suggest that the concentrations of these oxidized derivatives of PAHs increase easily in animal bodies. Recently, we have found that several PAHQs show oxidative damage to cellular components and DNA by producing reactive oxygen species (ROS) in the redox cycling.<sup>12)</sup> However, it is unclear whether PAHQs have endocrine-disrupting activities. The purpose of this study was to estimate estrogenic/antiestrogenic activities by the yeast two-hybrid assay and binding affinity of PAHQs by polarized fluorescence detection. Based on the results,

we discuss the relationship between structures and activities of quinoid PAHs.

## MATERIALS AND METHODS

**Chemicals**— 1,2-Naphthoquinone (1,2-NQ), acenaphthoquinone (AcQ), 1,4-phenanthrenequinone (1,4-PhQ), 9,10-PhQ, 1,4-anthraquinone (1,4-AQ), 9,10-AQ, 1,4-chrysenequinone (1,4-ChQ), 1,2-benzanthraquinone (1,2-BAQ), benzo[*c*]phenanthrene[1,4]quinone (BcPh-1,4-Q), BcPh-5,6-Q, benzo[*a*]pyrene[1,6]quinone (BaP-1,6-Q), BaP-3,6-Q, BaP-4,5-Q, BaP-6,12-Q, BaP-7,8-Q, BaP-7,10-Q, BaP-11,12-Q, 5,6,8,9-tetrahydrobenzo[*a*]anthracen-11[10H]-one (BaA-11-one), benzo[*a*]fluoren-11-one (BaFl-11-one), benzo[*b*]fluoren-11-one (BbFl-11-one) and 1-hydroxy-9-fluorenone (1-OH-Fl-9-one) were from Chiron AS (Trondheim, Norway). 3,4-Dihydrobenzo[*a*]anthracen-1[2H]-one (BaA-1-one) and 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one (BaP-7-one) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). 9-Fluorenone, anthrone and benzanthrone were purchased from Tokyo Chemical (Tokyo, Japan). Structures and abbreviations of test compounds are shown in Figs. 1 and 2. Test compounds were dissolved in ethanol and stored at  $-20^\circ\text{C}$  until use. All other chemicals were of the highest quality available from commercial sources.

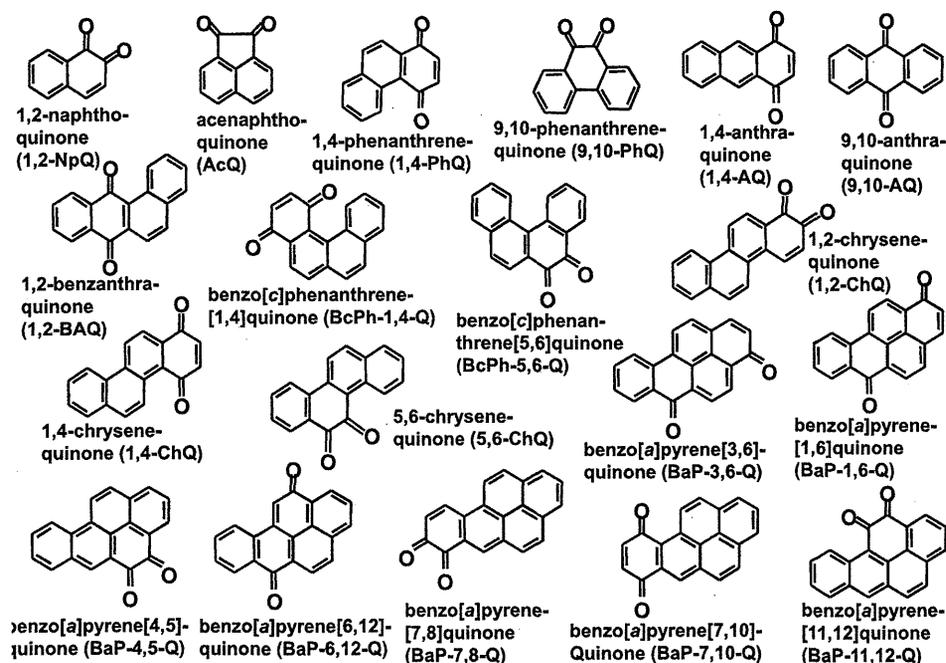


Fig. 1. Structures and Abbreviations of PAHQs

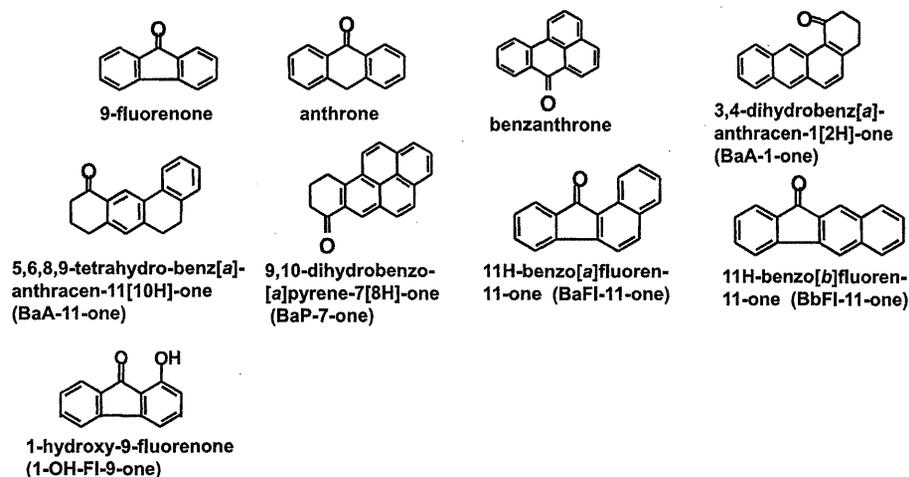


Fig. 2. Structures and Abbreviations of Ketone PAHs

### Assay of Estrogenic and Antiestrogenic Activities

— Estrogenic and antiestrogenic activities of PAH derivatives were evaluated by the yeast two-hybrid assay method using yeast cells expressing human ER $\alpha$  according to our previous reports.<sup>7,9)</sup> To examine the agonistic activity, the yeast cells were grown overnight at 30°C with shaking in synthetic defined medium free from tryptophan and leucine, and treated with each test compound in the concentration range from  $1 \times 10^{-9}$  M to  $1 \times 10^{-6}$  M at 30°C for 4 hr. After the incubation, the treated cells were collected and enzymatically digested with 1 mg/ml Zymolyase 20 T at 37°C for 30 min. 2-Nitrophenyl- $\beta$ -D-galactoside was added to the lysate to a final concentration of 4 mg/ml. After incubation at 30°C for 45 min, the reaction was stopped by the addition of 1 M Na<sub>2</sub>CO<sub>3</sub>. The yeast debris was removed by centrifugation and  $\beta$ -galactosidase activity was assayed by measuring the absorbance of supernatant at 415 nm. The data were representative of three independent experiments. Relative effective potency of estrogenic activity (REP<sub>E</sub>) was calculated as the inverse value of the relative concentration of the test compound that gave the same activity of E<sub>2</sub>. Relative effective potency of antiestrogenic activity (REP<sub>AE</sub>) was calculated as the inverse value of the relative concentration of the test compound that gave the same activity of 4-hydroxytamoxifen (4-OHT) in the presence of 1 nM E<sub>2</sub>.

**Receptor Binding Assay**— The assay was performed by using a PanVera P2698 ER $\alpha$  Competition Assay, Green (Takara Bio, Otsu, Japan). Fourteen  $\mu$ l of Fluormone<sup>TM</sup> ES2 (FES2) and 14  $\mu$ l of

ER $\alpha$  were mixed with 2672  $\mu$ l of screening buffer (SB) in a glass tube. The mixture was settled for 1 hr at room temperature to form FES2-ER $\alpha$  complex. Then, 50  $\mu$ l of the complex solution was transferred from the tube into a 96-well plate. Forty-eight  $\mu$ l of SB and 2  $\mu$ l of test solution were successively added to the complex solution in the well and the contents in the well were mixed. After settling the plate for 1 hr at room temperature, the polarized fluorescence intensity of the solution was monitored by a Fluplo monitor (Takara Bio). The control solution was a mixture of 98  $\mu$ l of SB and 2  $\mu$ l of dimethyl sulfoxide (DMSO). Positive control solution was a mixture of 48  $\mu$ l of SB, 50  $\mu$ l of the complex solution and 2  $\mu$ l of DMSO. Negative control solution was a mixture of 48  $\mu$ l of SB, 50  $\mu$ l of the complex solution and 2  $\mu$ l high-concentrated ( $\geq 1 \times 10^{-5}$  M) E<sub>2</sub> solution, respectively. Relative binding affinity (RBA) was calculated as the inverse value of the relative concentration of the test compound that gave the same competition activity of E<sub>2</sub> in the presence of FES2.

**Calculation of Physical Parameters**— In order to estimate the structural characteristics of PAH derivatives used, the following physical parameters were used. The L/B ratio of the rectangular van der Waals plane surrounding each mother PAH molecule was calculated with a molecular modeling program (CACHeworksystem ver.4.1.1 for Apple Macintosh, Fujitsu Co. Ltd., Chiba, Japan) using the L and B values obtained from the Polycyclic Aromatic Hydrocarbon Structure Index.<sup>13)</sup> The O-H distance, the distance between the oxygen atom of the carbonyl group and the hydrogen atom located

farthest from the carbonyl group was calculated by using a computer with a molecular modeling program. Other conditions for calculating physical parameters were the same as in our previous report.<sup>9)</sup>

## RESULTS

### Estrogenic Activity

An increase of  $\beta$ -galactosidase activity was observed for several PAHQs such as BaP-3,6-Q ( $REP_E = 2.3 \times 10^{-4}$ ), 1,2-ChQ ( $REP_E = 0.5 \times 10^{-4}$ ) and BaP-7,8-Q ( $REP_E = 0.2 \times 10^{-4}$ ) (Table 1). However, the activities of these quinines were much weaker than those of 4-OHBaA ( $REP_E = 7.5 \times 10^{-3}$ ) which showed the strongest estrogenic activity among OHPAHs.<sup>9)</sup> The other PAHQs did not

show estrogenic activity. None of the ketone PAHs showed estrogenic activity at concentrations between  $1 \times 10^{-9}$  M and  $1 \times 10^{-6}$  M.

### Antiestrogenic Activity

Significant decreases of  $\beta$ -galactosidase activity were observed for 1,4-PhQ, BcPh-5,6-Q, 1,2-ChQ, 1,4-ChQ, 5,6-ChQ, BaP-1,6-Q, BaP-4,5-Q, BaP-7,8-Q, BaP-7,10-Q and BaP-11,12-Q (Table 1). Among them, 1,4-ChQ and 5,6-ChQ exhibited the strongest antagonistic effect ( $REP_{AE} = 0.97$ ). Their activity of these two quinines was almost as strong as that of 4-OHT and about a half of 3-OHBcPh ( $REP_{AE} = 1.9$ ) which showed an antagonistic effect in our previous report.<sup>9)</sup> None of the ketone PAHs showed antiestrogenic activity at concentrations between  $1 \times 10^{-9}$  M and  $1 \times 10^{-6}$  M.

Table 1. Estrogenic/Antiestrogenic Activities and Receptor Binding Affinities

Type <sup>a)</sup>	Abbreviation	$REP_E^b, \times 10^{-4}$	$REP_{AE}^c$	RBA <sup>d)</sup>
Q-2	1,2-NQ	< 0.01	< 0.01	0.94
Q-3	AcQ	< 0.01	< 0.01	0.11
	1,4- PhQ	< 0.01	0.65	0.78
	9,10-PhQ	< 0.01	< 0.01	0.01
	1,4- AQ	< 0.01	< 0.01	0.72
	AQ	< 0.01	< 0.01	0.14
Q-4	1,2-BAQ	< 0.01	< 0.01	0.13
	BcPh-1,4-Q	< 0.01	< 0.01	1.07
	BcPh-5,6-Q	< 0.01	0.76	0.33
	1,2-ChQ	0.5	0.50	0.93
	1,4-ChQ	< 0.01	0.97	1.03
	5,6-ChQ	< 0.01	0.97	0.81
Q-5	BaP-1,6-Q	< 0.01	0.65	0.56
	BaP-3,6-Q	2.3	< 0.01	0.84
	BaP-4,5-Q	< 0.01	0.69	0.11
	BaP-6,12-Q	< 0.01	< 0.01	—
	BaP-7,8-Q	0.2	0.60	0.96
	BaP-7,10-Q	< 0.01	0.50	0.85
	BaP-11,12-Q	< 0.01	0.42	0.78
K-3	9-Fluorenone	< 0.01	< 0.01	0.29
	Anthrone	< 0.01	< 0.01	0.26
	1-OH-FI-9-one	< 0.01	< 0.01	—
K-4	Benzanthrone	< 0.01	< 0.01	0.25
	BaA-1-one	< 0.01	< 0.01	0.48
	BaA-11-one	< 0.01	< 0.01	—
K-5	BaP-7-one	< 0.01	< 0.01	0.02
	BaF-11-one	< 0.01	< 0.01	0.17
	BbF-11-one	< 0.01	< 0.01	0.33

a) Q, quinoid; K, ketone; numbers indicate number of rings. b) Estrogenic activity of each test compound was measured by the yeast-two hybrid assay.  $REP_E$  was calculated from the value of  $E_2$  as a positive control. c) Antiestrogenic activity of each test compound was measured by the yeast-two hybrid assay.  $REP_{AE}$  was calculated from the value of 4-OHT as a positive control. d) Receptor binding affinity was monitored by the polarized fluorescence method. RBA was calculated as the inverse value of the relative concentration of the test compound that gave the same competition activity of  $E_2$  in the presence of FES2.

Among OHPAHs tested in our previous work, hydroxylated derivatives of BaA, BcPh and BaP, which have 4 and 5 rings, respectively, showed antiestrogenic activity. However, PAHQs showed a different profile. Quinoid derivatives of Ph, BcPh, Ch and BaP having 3–5 rings showed antiestrogenic activity.

### Binding Affinity

Strong binding affinity ( $RBA \geq 0.56$ ) was observed for 1,2-NQ, 1,4-PhQ, 1,4-AQ, BcPh-1,4-Q, 1,2-ChQ, 1,4-ChQ, 5,6-ChQ, BaP-1,6-Q, BaP-3,6-Q, BaP-7,8-Q, BaP-7,10-Q and BaP-11,12-Q. Among them, BcPh-1,4-Q and 1,4-ChQ showed the strongest affinities which were comparable to that of  $E_2$ . It should be noted that all PAHQs which showed estrogenic/antiestrogenic activities had  $RBA \geq 0.18$ . The RBA values of ketone PAHs tested were not strong. The RBA values were in the range from 0.17 to 0.48. RBA of each test compound is given in Table 1.

## DISCUSSION

Among 28 PAH derivatives having 2–5 rings, only three quinones, BaP-3,6-Q, 1,2-ChQ and BaP-7,8-Q showed estrogenic activity. However, their activities were much weaker than those of estrogenic OHPAHs. Therefore, in the following discussion, only BaP-3,6-Q was used as an estrogenic PAHQ. On the other hand, 10 PAHQs having 3–5 rings exhibited antiestrogenic activity ( $REP_{AE} \geq 0.42$ , Table 1). The activities of 1,4-ChQ and 5,6-

ChQ were about 1/2 of the activity of 3OHBcPh ( $REP_{AE} = 1.9$ ) but comparable to the activity of 2-OHBcPh ( $REP_{AE} = 0.69$ ).<sup>9</sup> Importantly, the data in Table 1 show that phenol group was not always necessary to exhibit estrogenic/antiestrogenic activities. However, ketone PAHs did not show estrogenic/antiestrogenic activities.

In order for OHPAHs to exhibit estrogenic/antiestrogenic activities, they must have an affinity for ER.<sup>9</sup> Furthermore, RBA is correlated with  $REP_E$  or  $REP_{AE}$ .<sup>14</sup> PAHQs fell into three groups (Fig. 3):  $RBA \geq 0.18$  and  $REP_{AE} \geq 0.42$  or  $REP_E = 2.3$  such as 1,4-ChQ and BaP-7,8-Q,  $RBA \geq 0.11$  and  $REP_{AE} = 0$  such as BcPh-1,4-Q and  $RBA = 0$  and  $REP_{AE} = REP_E = 0$  such as 9,10-PhQ. None of the PAHQs had  $RBA = 0$  but  $REP_{AE} \geq 0.42$ . Group 1 contained 10 PAHQs, although a parallel relationship was not observed between RBA and  $REP_{AE}$ . As an interaction of PAHQs in Group 2, such as 1,2-NQ, may interact with ER through covalent bonding to thiol groups of the protein. The data in Fig. 3 suggest that RBA is necessary but not sufficient for PAHQs to exhibit estrogenic/antiestrogenic activities. Several PAHQs such as 1,2-NQ, 1,4-AQ and 1,4-ChQ can covalently bind to macromolecules through reactive thiol residues.<sup>15</sup> Among these three PAHQs, both 1,2-NQ and 1,4-AQ were in Group 2 which did not show antiestrogenic activity. 1,4-ChQ only was in Group 1 which showed both strong binding affinity and strong antiestrogenic activity, suggesting that the covalent binding affinity of 1,4-ChQ may contribute to its strong antiestrogenic activity.

These results suggest that active PAHQs would pass through the cell membrane and bind to ER in the nucleus. Although the  $P_{o/w}$  values of PAHQs having 3–5 rings are smaller than those of the corresponding OHPAHs (for example, the computer-calculated log P values of 1,4-ChQ, and 1-OHCh were 2.459 and 4.769) the hydrophobicity of PAHQs might be enough to pass through the cell membrane.

The data in Table 1 showed that 11 estrogenic or antiestrogenic PAHQs have 3–5 rings. We previously found that estrogenic OHPAHs had the narrow range of L/B ratios and that antiestrogenic OHPAHs had narrow range of OH distances which made it easier to bind to the active site of ER.<sup>9</sup> The computer-calculated L/B ratios and O-H distances of PAHQs tested are plotted in Fig. 4. The L/B ratios of active PAHQs are in the range from 1.27 (BaP-1,6-Q) to 1.41 (BaP-7,10-Q) and in the range from

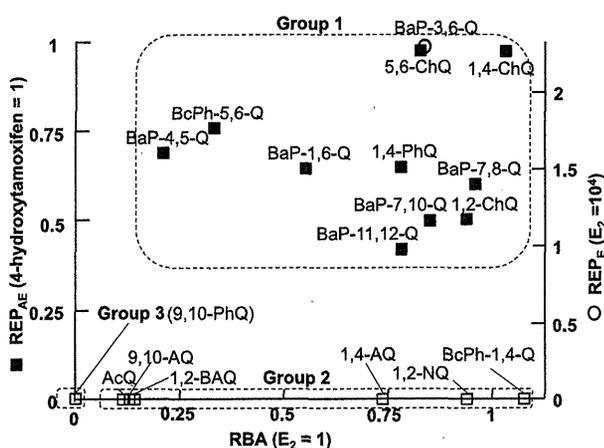


Fig. 3. Relationship between RBA and  $REP_{AE}$  or  $REP_E$  of PAHQs  
 ■, antiestrogenic; ○, estrogenic; □, inactive.

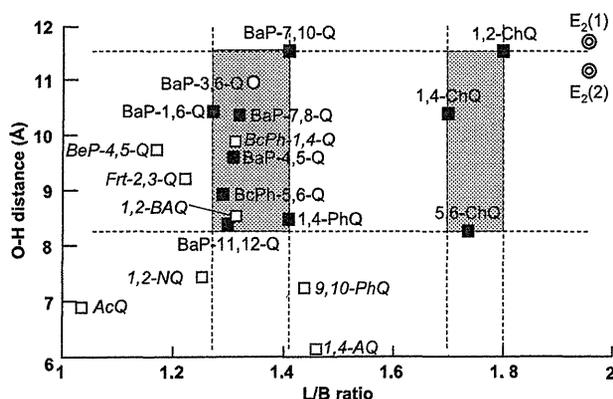


Fig. 4. Relationship between O-H Distance and L/B Ratio of PAHQs  
 ■, antiestrogenic; ○, estrogenic; □, inactive, ⊙, E<sub>2</sub>.

1.7 (1,4-ChQ) to 1.8 (1,2-ChQ). The former range contains 8 quinoid derivatives of BaP, BcPh and Ph including estrogenic BaP-3,6-Q and the latter contains 3 quinoid derivatives of Ch. Considering that the L/B ratio of E<sub>2</sub> was 1.95 and PAHQs having 6 rings or more were not tested in this work, L/B = 1.8 might not be the upper limit. On the other hand, the O-H distances of estrogenic/antiestrogenic PAHQs were in the range from 8.2 Å (5,6-ChQ) and 11.5 Å (1,2-ChQ). This range was close to the values of O-O distances of E<sub>2</sub> (11.7 Å and 11.2 Å). The values of inactive PAHQs were outside these areas with two exceptions, 1,2-BAQ and BcPh-1,4-Q.

The phenol group (OH-3) of E<sub>2</sub> makes hydrogen bonds with glutamic acid (Glu)353 and arginine (Arg)394 of ER and H<sub>2</sub>O and the alcohol group (OH-17) of E<sub>2</sub> has an affinity for the nitrogen atom of histidine 524 of ER. In these hydrogen bonds, the distance between Glu353 and the phenol group was the shortest (2.37 Å), which might be the strongest bond. On the other hand, there is a van der Waals interaction between E<sub>2</sub> and the binding site of ER.<sup>16,17</sup> Both the hydrogen bonding and van der Waals interaction were also necessary for OHPAHs to exhibit activity. Although PAHQs have no phenol groups, the carbonyl groups might play a role in the interaction with ER. Hydrogen bonds can be formed between one or two carbonyl groups of PAHQ and Glu353 and Arg394 of ER. According to the binding scheme of E<sub>2</sub> and ER [Fig. 5 (a)],<sup>9</sup> ER can bind to 1,4-ChQ, 1,2-ChQ and BaP-7,8-Q [Fig. 5 (b)–(d), respectively]. BcPh-1,4-Q and 1,2-BAQ were not antiestrogenic even though their L/B ratio and O-H distance were in the area of active PAHQs. This may be because the formation of hy-

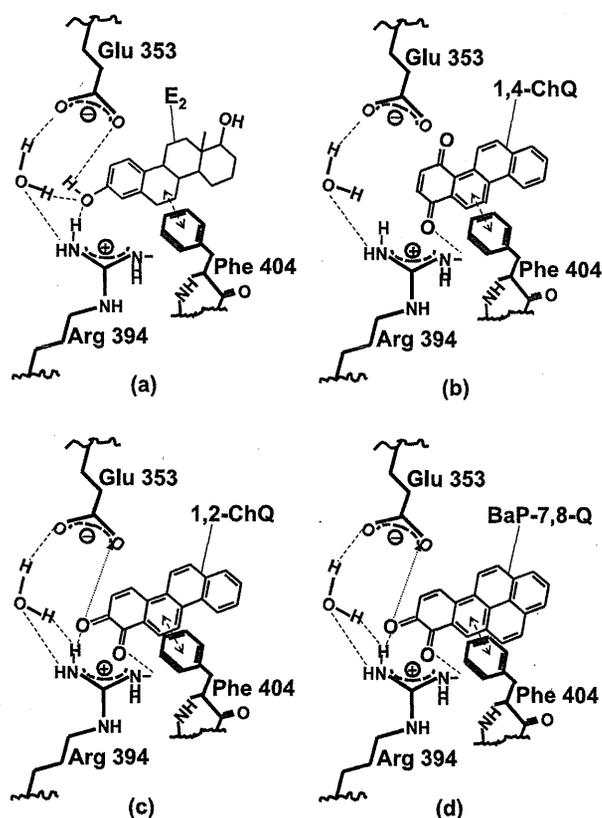


Fig. 5. Binding between E<sub>2</sub> and ER and Possible Bindings between PAHQ and ER  
 (a) E<sub>2</sub>; (b) 1,4-ChQ; (c) 1,2-ChQ.

drogen bond may be disturbed by aromatic rings surrounding the carbonyl groups of BcPh-1,4-Q and 1,2-BAQ.

Our results lead to three conclusions. Several PAHQs having 3–5 rings showed antiestrogenic activities. The most strongly antiestrogenic PAHQs tested were 1,4-ChQ and 5,6-ChQ, followed by BcPh-5,6-Q, BaP-4,5-Q, 1,4-PhQ, BaP-1,6-Q, BaP-7,8-Q, BaP-7,10-Q, BaP-11,12-Q and 1,2-ChQ. BaP-3,6-Q showed estrogenic activity. The other PAHQs and ketone PAHs did not show estrogenic/antiestrogenic activities. Several physical parameters such as L/B ratio and O-H distance of antiestrogenic/estrogenic PAHQs were in narrow ranges, suggesting a structure-activity relationship. The interaction between active PAHQs and ER may be due to hydrogen bonding between carbonyl groups and amino acid residues and van der Waals forces.

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