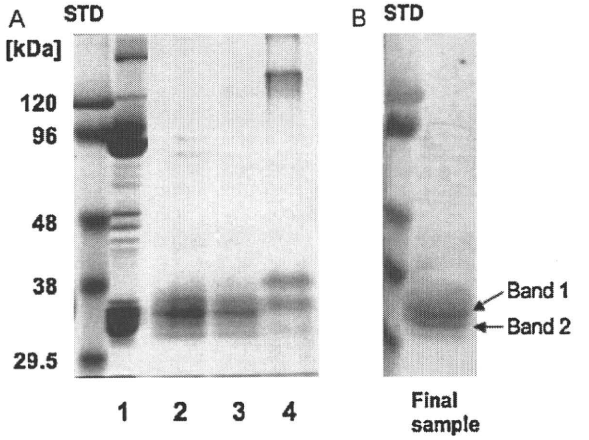

Fig. 1. Purification of rTBT-bp1 detected by SDS–PAGE. (A) Detection of His-tagged rTBT-bp1 fractionated by affinity chromatography: lane 1, flow-through material; lane 2, eluate at 50 mM imidazole; lane 3, eluate at 150 mM imidazole; lane 4, eluate at 450 mM imidazole. (B) Detection of final sample of His-tagged rTBT-bp1 fractionated by gelfiltration chromatography. STD is molecular weight standard.

Table 2
Results of N-terminal amino acid sequence analysis. Band 1 and 2 are upper and lower band of final sample, respectively.

	N-terminal amino acid sequence
Band 1 (upper)	APTPEETSQLVSPVS
Band 2 (under)	APTPEEXXQLV

3.2. TBT binding assay

A binding assay indicated that rTBT-bp1 bound TBT. After incubation of the rTBT-bp1 solution (38 μM) with TBT solution (380 μM) at 15 °C for 16 h, the mixture was fractionated by gel filtration chromatography. rTBT-bp1 was detected in fractions 18 and 23, and the peaks for TBT were corresponded to those for the protein (Fig. 3). This result indicated that rTBT-bp1 bound TBT because the molecular weight of rTBT-bp1 and TBT were 35 kDa and 289 Da, respectively. The molar ratios of rTBT-bp1 to TBT in fractions 18 and 23 were 6:1 and 4:1 (rTBT-bp1:TBT), respectively. The molecular weights of rTBT-bp1 estimated from the elution times were 27 kDa for fraction 18 and 3.2 kDa for fraction 23. These values did not agree with the values determined by means of SDS–PAGE analysis (*i.e.*, 30 and 35 kDa).

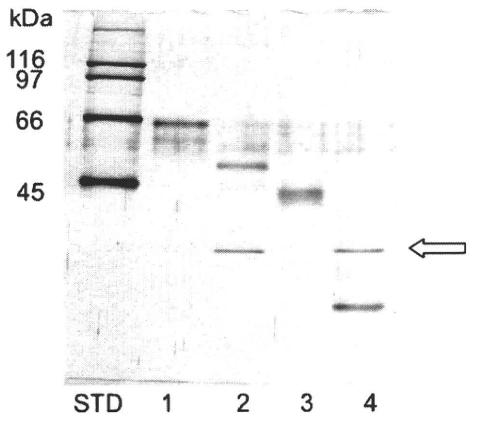

Fig. 2. SDS–PAGE of purified and deglycosylated TBT-bp1: lane 1, control glycoprotein; lane 2, deglycosylated control glycoprotein; lane 3, purified TBT-bp1; lane 4, deglycosylated TBT-bp1. The arrow indicates glycosidase F. STD is molecular weight standard.

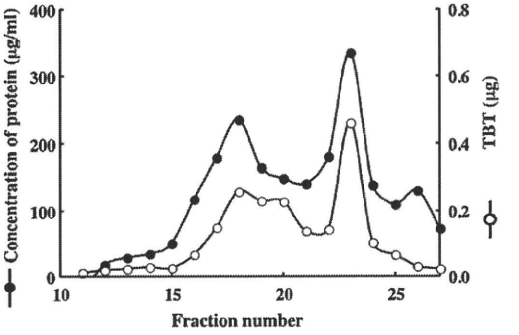

Fig. 3. Elution profile of mixture containing rTBT-bp1 and TBT obtained by gel filtration chromatography on a TSK gel G2000SWxl column.

3.3. In vitro assay of restoration of osteoblastic activity inhibited by TBT in cultured scales of goldfish

The results of the assay for the ability of rTBT-bp1 (10^{-7} M) to restore osteoblastic activity inhibited by TBT are shown in Fig. 4A. The osteoblastic activities in the scales of the TBT treatment group significantly lower than those of the control (10^{-8} M: $P=0.032$; 10^{-7} M: $P=0.006$; 10^{-6} M: $P=0.0006$). In contrast, osteoblastic activities were recovered in the group of co-treated with rTBT-bp1 (10^{-7} M) and 10^{-8} or 10^{-6} M TBT, although at 10^{-6} M TBT osteoblastic activity remained significantly inhibited (Fig. 4B, $P=0.036$). These results indicate that rTBT-bp1 restored osteoblastic activity inhibited by TBT.

The results of a similar assay conducted with nTBT-bp1 (10^{-7} M) are shown in Fig. 4C. Osteoblastic activity was restored in all the groups co-treated with nTBT-bp1 (10^{-7} M) and TBT (10^{-8}, 10^{-7}, or 10^{-6} M). The nTBT-bp1 restored osteoblastic activity as well or better than rTBT-bp1.

4. Discussion

We obtained histidine-tagged rTBT-bp1 by means of baculovirus gene expression system in silkworm larvae (Fig. 1). The predicted molecular weight of rTBT-bp1, which consists of 280 amino acid residues, is approximately 30 kDa. This value corresponds to the molecular weight calculated for the lower band (band 2) observed on the SDS–PAGE. However, the molecular weight of the upper band (band 1) was estimated to be 35 kDa. The difference between the molecular weight of the two bands might be due to glycosylation modification in the endoplasmic reticulum of the silkworm cells. nTBT-bp1 purified from blood of Japanese flounder is reported to be 42% glycosylated with N-linked sugar chains (Shimasaki et al., 2002). In the expression system using silkworm larvae, some recombinant glycoprotein is expressed in various glycoforms (Wu et al., 2002; Kato and Park, 2007). Kato and Park reported that the signal peptide might relate to glycosylation of two types of fusion proteins produced in Bm5 cells. In our expression system, the signal peptide of TBT-bp1 originating from Japanese flounder would have contributed to these two types of rTBT-bp1.

The TBT binding assay strongly suggests that rTBT-bp1 retains a binding ability by folding into the same structure as nTBT-bp1. Shimasaki et al. (2002) reported that the binding ratio of nTBT-bp1 to TBT is 30:1.

In gel filtration analysis, rTBT-bp1 and TBT co-eluted in the same two fractions 18 and 23. SDS–PAGE analysis indicated that molecular weights of proteins eluted during the gel filtration analysis were

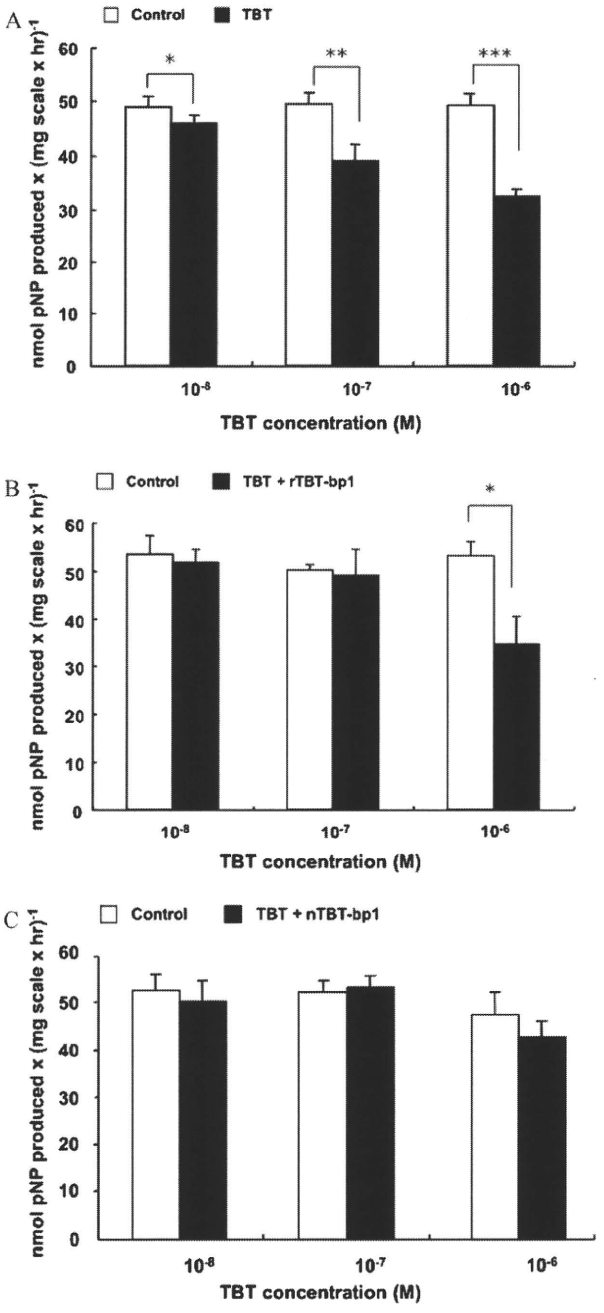

Fig. 4. Effects of (A) TBT, (B) TBT puls rTBT-bp1, and (C) TBT plus nTBT-bp1 on osteoblastic activity in cultured goldfish scales after 6 h of incubation. The alkaline phosphatase (ALP) activity was determined as a mesure of osteoblastic activity. *, **, and *** indicate statistically significant difference to the level of activity in the control scales, at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

30 and 35 kDa, respectively. These were confirmed to be rTBT-bp1 by N-terminal amino acid analysis. However, molecular weights of 27 kDa and 3.2 kDa were calculated for fractions 18 and 23, respectively. The result of gel filtration of glycoprotein tends to change according to its carbohydrate content, shape, adsorption and so on (Andrews, 1965). The rTBT-bp1 proteins in both fraction bound TBT, despite the proposed difference in glycosylation. This result suggests that glycosylation of TBT-bp1 did not contribute to the protein's ability to bind TBT.

The addition of rTBT-bp1 or nTBT-bp1 with TBT exerted the recovery of osteoblastic activity inhibited by a single exposure to TBT at 10^{-7} M (Fig. 4). It is suggested that TBT-bp1 binds to TBT and reduces its toxic effects on scale calcification or on bone tissues that results in malformation. In medaka larvae, it was demonstrated that the malformation of the spinal marrow and tail fin might be caused by TBT that has been maternally transferred to the embryo (Hano et al., 2007). In adult fish or mammals, there have been no reports of TBT induction of abnormal bone formation. Malformation of bone during developmental stage induced by TBT might arise from low levels of endogenous TBT-bp1 resulting in the inhibition of calcification. Further experiments are needed to clarify the expression of the TBT-bp1 gene during development.

The ability of rTBT-bp1 to restore the osteoblastic activity was lower than that of nTBT-bp1, even though it was demonstrated that rTBT-bp1 retained a higher binding ability than nTBT-bp1. The reason of this difference between recombinant and native protein is not clear. However, we expect that TBT-bp1 has another mechanism for TBT binding to protect the scales using for example sugar chain recognition.

TBT-bps in fish may generally respond to exposure of chemicals. Thirteen putative TBT-bp-like proteins have been found in eight species of teleost including medaka, zebrafish (*Danio rerio*), and spotted green pufferfish (*Tetraodon nigroviridis*) (Satone et al., 2008). In the brain of Japanese medaka, the expression levels of two types of genes encoding TBT-bp-like proteins are altered by polychlorinated biphenyls (Volz et al., 2005). The expression levels of mRNA for TBT-bp1 were up regulated in medaka liver exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Nakayama et al., 2008). Further experiments are required to reveal the binding ability of TBT-bps to chemicals.

In conclusion, we purified rTBT-bp1 and confirmed that it bind to TBT. We found that TBT-bp1 bind to TBT in the body of fish and restored osteoblastic activity inhibited by TBT. This report related to protective function of TBT-bp1 on fish body surface will be valuable to future experiments investigating physiological functions of TBT-bps and their homologs.

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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．^{1}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いるが，^{1}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，^{1}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が限られる．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

キーワード：定量NMR，qNMR，純度，トレーサビリティ
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Title: How to determine the absolute purities of target organic compounds? − Development of quantitative NMR (qNMR) −
Author: Naoki Sugimoto

同位体希釈質量分析法があるが，こちらも分析できる物質の量が限られており，一般に分析の迅速性に欠ける．

市場に流通する「実用標準物質」は，「国家標準物質（NMIJ CRM）」を介して同じ物質同士の校正の連鎖で SI トレーサビリティを確保している（計量標準供給制度（JCSS: Japan Calibration Service System））．「国家標準物質」は SI にトレーサブルな方法である上述の「一次標準測定法」を利用して厳密に値付けを行っている．この「国家標準物質」を利用して「二次標準物質」，次いで「実用標準物質」を段階的に校正しつつ量的な拡大を行い，市場に要求される量を供給している（図 1 左）．この標準物質供給システムは必ず同じ物質の上位標準を 1 対 1 の関係で参照して SI へのトレーサビリティを確保している．従って，滞りなく段階的な校正が行われ，最終的に「実用標準物質」＝「市販標準品」の関係が成り立っていれば，その純度値について疑うべき問題は全くない．しかしながら，現在，ケミカルアブストラクトには 5000 万以上の化合物が収録されており，分析・評価対象となり得る化合物が仮に 1000 分の 1 としても 5 万以上となり，これらすべてについて上位標準となる「国家標準物質」の作製がコスト面から到底実現できないことが想像できるだろう．一方，分析・評価対象となる有機化合物の標準品や試薬の供給について市場の要求は絶え間なくあるため，結果として，それぞれの試薬会社はその要求に応えるべく，各社独自の方法，たとえば，比吸光度あるいはクロマトグラム上で観察される主ピークの面積百分率などから純度や含量を品質保証の目的で値付けて供給している．すなわち，ほとんどの市販標準品や試薬は，「国家標準物質」を上位標準とした標準物質供給システムで値付けされては供給されていない．従って，SI トレーサビリティの連鎖から完全に外れているため，極端に言えば，これらに表示された純度値を保証する根拠はないに等しいということになる．これが市販標準品や試薬の純度や含量値に関するサイレントで大きな問題である．

以上のことから，有機化合物の市販標準品や試薬の絶対純度の新規測定法の開発と SI トレーサビリティを確保した新たな標準物質供給システムの構築が要求されている．qNMR は「一次標準測定法」のうち，「一次比率法」の資格を原理的に有する簡便且つ迅速な分析法であると共に，これまでの標準物質供給システムを刷新する手法として国内外の研究機関および試薬会社より注目され始めている（図 1 右）．

2.　定量 NMR（quantitative NMR: qNMR）の原理(1, 2)

有機化合物は炭素，水素，酸素などが共有結合した分子である．異なる分子構造で異なる物性値を持つ分子であっても，構成する最小単位の同種の原子同士は等価である．qNMR は，この原子レベルの比較分析である．定量用に最適化された ^{1}H-NMR では，化学シフトの異なる各シグナルの面積比は，分子上の各炭素に結合した水素原子の数の比に対応し，測定対象の化合物の分子構造に関わらず，すべて水素原子が定量的な信号として観測される．このことから，化合物 A の特定の置換基上の水素のシグナル面積（I_A）は，置換基上の水素の数（H_A）×化合物 A のモル濃度（m_A）として観察される（式 1 の分子）．別の分子構造の化合

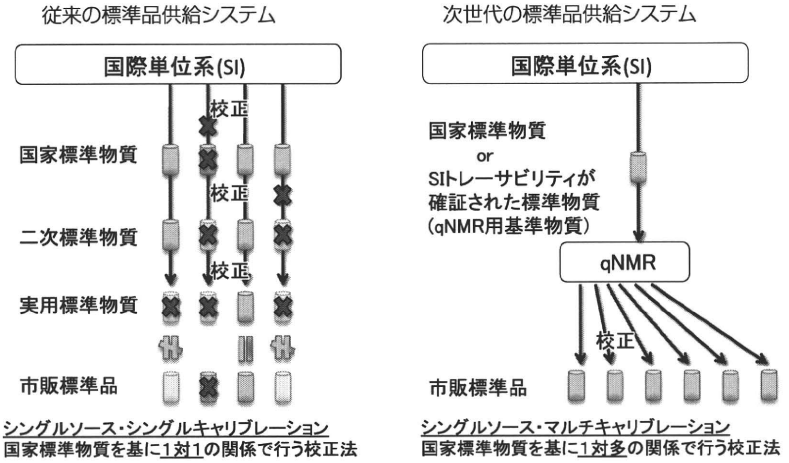

図 1　従来の標準物質供給システムと qNMR による SI トレーサビリティ体系の再構築
qNMR では，原理的に 1 つの上位標準を介した校正により，無限の有機化合物の市販標準品の純度を評価・供給することが可能．

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測定対象のあらゆる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）．

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

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

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-スルフォン酸-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

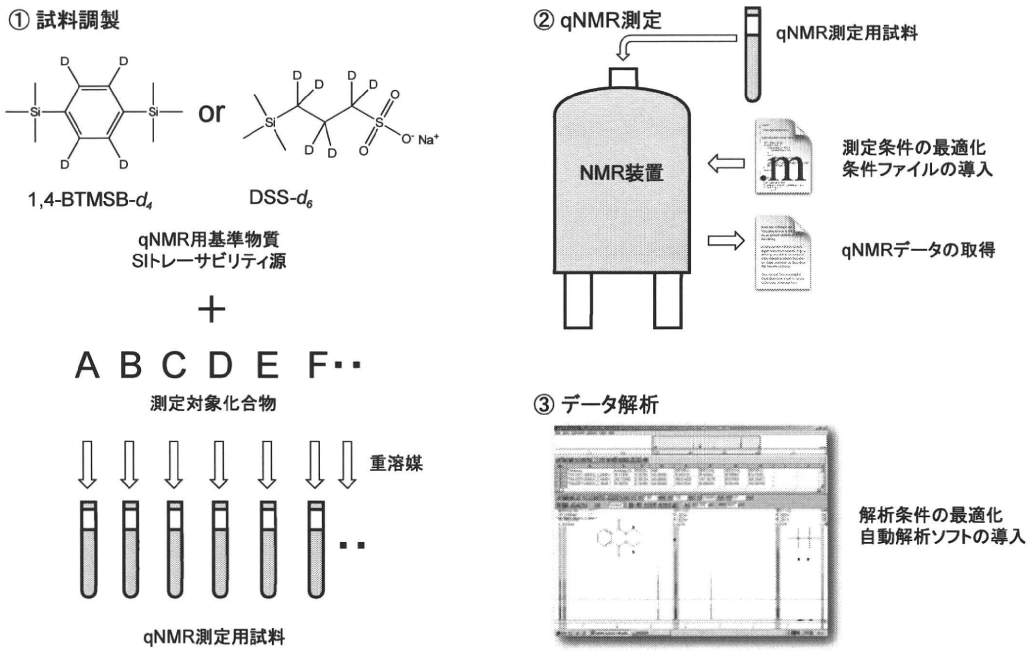

図2 SIトレーサビリティを確保したqNMRによる絶対純度測定法の実際の手順

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収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

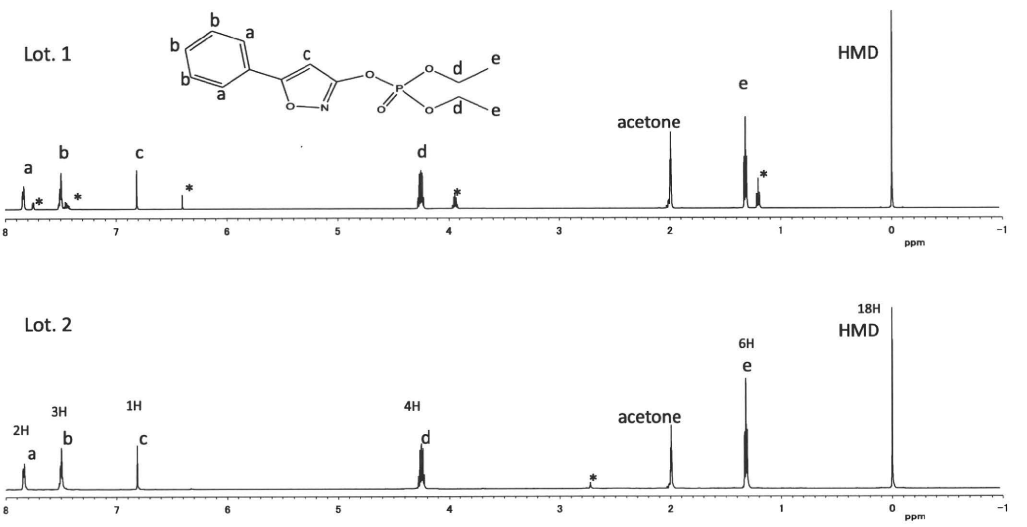

図3　残留農薬試験用イソキサチオンオキソン（IXO）標準品（ロット1, 2）の qNMR スペクトル
qNMR 用に測定条件を最適化した JNM-ECA（600 MHz）を用いた．重溶媒＝アセトン-d_6，基準物質＝ヘキサメチルジシラン（HMD）（$C_6H_{18}Si_2$）．HMD と IXO の a～e のシグナル強度比より絶対純度を算出した．a～e は各シグナルの帰属．＊は分解物または不純物に由来するシグナル．

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．今後，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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ARTICLE

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

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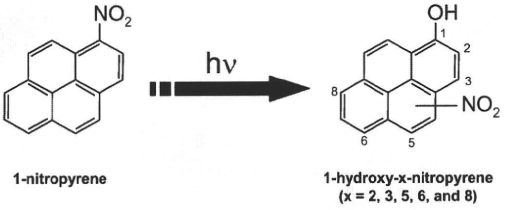
Supporting Information

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 mono-nitro-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 ΣOHNP (=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 ΣOHNP/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).[1] 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_2,[2] and 2-nitrofluoranthene is formed via two pathways, i.e., OH or NO_3 radical-initiated reactions in the gas phase.[2] 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.[3] 1-NP taken up by humans and animals is transformed to various metabolites such as hydroxynitropyrenes (OHNPs) in the presence of cytochrome P450 enzymes.[4] 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[5] and diesel exhaust particles (DEPs).[3,6,7] Several studies have found that most OHNP isomers have lower mutagenic activity than the parent 1-NP.[7−9] 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,[10] 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,[11−13] benzene,[13,14] acetonitrile,[13,15] dimethyl sulfoxide (DMSO),[16,17] 2-propanol,[13,18] diisooctylphthalate,[19] carbon tetrachloride,[13] 3-methylpentane,[13] toluene,[13] ethanol,[13] hexane,[13] and cyclohexane[13,20]) and on solid substrates (e.g., glass plates,[21] silica,[16] coal fly ash,[22] cellulose,[12] diesel soot particles,[23] and wood smoke particles[23]). The products of these reactions include OHNPs such as 1-hydroxy-2-nitropyrene (1-OH-2-NP),[11] 2-hydroxy-1-nitropyrene (2-OH-1-NP),[15] and 9-hydroxy-1-nitropyrene (9-OH-1-NP)[14] as well as 1-hydroxypyrene (OHPy),[11] pyrenediones,[17,21] and pyrene.[11] So far, only one of these OHNPs, 9-OH-1-NP, has been

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detected in airborne particles.[14] Although 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP have been observed in ambient samples,[5] 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.[24] However, the organic phase of ambient aerosols is very complex, and the composition of it remains very uncertain.[19] 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 ± 2 K. The 1×10^{-5} mol L^{-1} 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 5.2×10^{16} photons cm^{-2} s^{-1}. 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. ^{1}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^{-1}, 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.[3] SRM 1975 was dissolved in methanol at an appropriate concentration.[25] The airborne particulate samples were prepared according to the previous report[26] (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.[26]

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

Analytical Instrumentation. LC/MS/MS analysis was performed as previously described[4] 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[26] (details are described in SI).

Details of ^{1}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.[4] 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.[27,28] Each OHNP isomer was purified by preparative normal phase HPLC (SUPELCO, Supelcosil PLC-SI, 21.2 mm i.d. × 250 mm, eluted with CH_2Cl_2 containing 0.5 mM CH_3COOH at 10 mL/min). To identify the synthetic compounds, their EI-MS and ^{1}H NMR spectra were compared with literature data.[8,11,28–30] 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_9) 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_3. 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_3 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

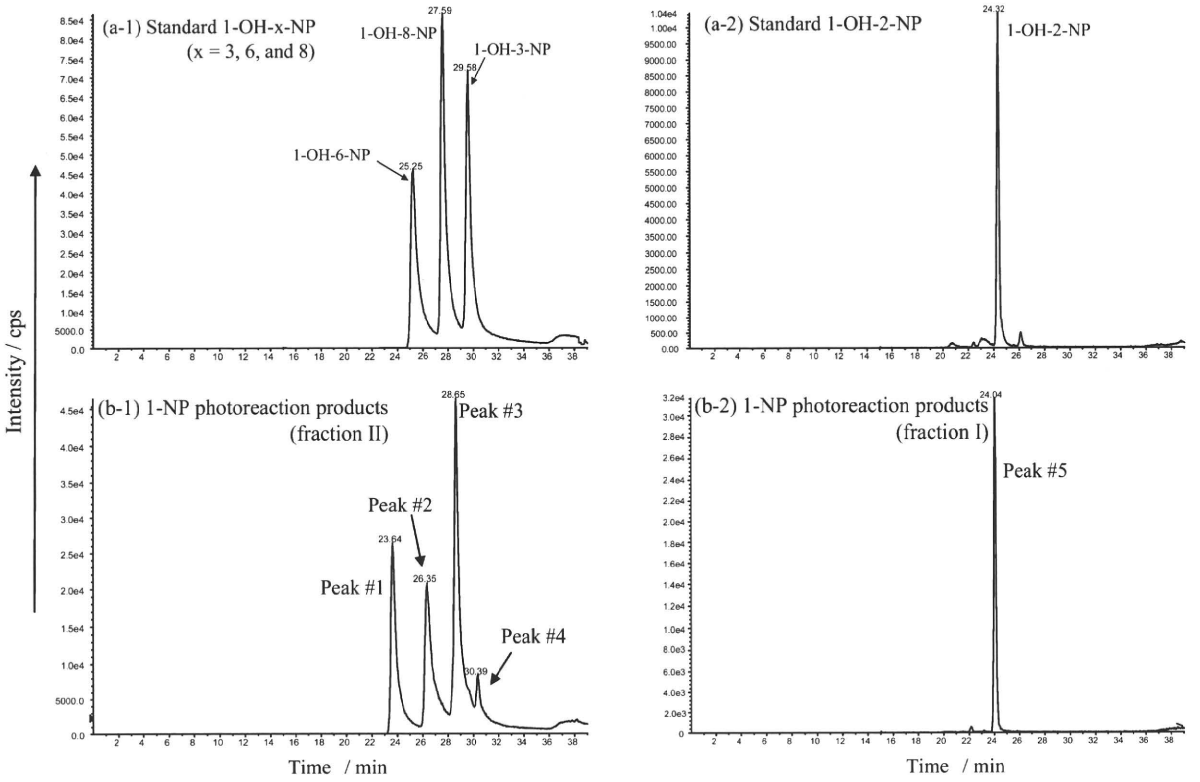

Figure 1. LC/MS/MS chromatograms for (a) standard solutions and (b) photoreaction products of 1-NP; m/z 262→232.

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 ^{1}H 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). ^{1}H NMR (acetone-d_6, δ 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 (Δ = +2.7 ppm). UV (methanol) λ_{max} (nm, rel. ε %): 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 ≥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.[14,15] 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.[14,15] Although 1-OH-5-NP, which is equivalent to 4-nitro-8-hydroxypyrene, has been reported to be a metabolite of 4-nitropyrene,[31] 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,

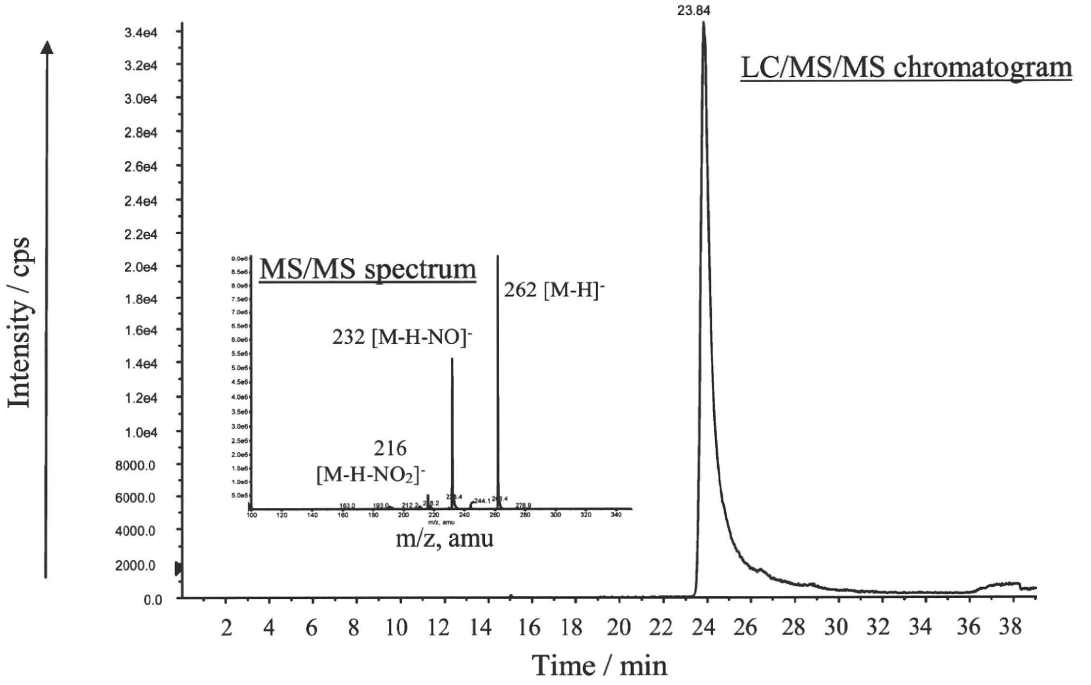

Figure 2. LC/MS/MS chromatogram (m/z 262→232) and MS/MS spectrum for fraction III of the 1-NP photoreaction product obtained by the HPLC fractionation.

Table 1. Concentrations of Hydroxynitropyrenes and 1-Nitropyrene and Concentration Ratio of Hydroxynitropyrenes to 1-Nitropyrene in Airborne Particles, SRM 1975, and SRM1650b[a]

	1-OH-2-NP	1-OH-3-NP	1-OH-5-NP	1-OH-6-NP	1-OH-8-NP	1-NP	ΣOHNP/1-NP[g]
airborne particles[b]	3.4[d]	0.9[d]	4.1	16.3	15.7	23.7	1.4
SRM1975[c]	n.d.	0.011	n.d.	0.030	0.019	18[e]	0.003
SRM1650b[c]	n.d.	0.09	n.d.	0.32	0.25	15[f]	0.04

[a] 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). [b] 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^{-3}. [c] Given in units of mg kg^{-1}. [d] Calculated using 0.5 of the minimum detectable values as concentrations lower than the quantification limits. See Table S2 for detail. [e] 112% of the value certified by NIST (16.4 ± 0.2 mg kg^{-1}). [f] 81% of the value certified by NIST (18.2 ± 0.2 mg kg^{-1}). [g] ΣOHNP = 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 $\mu g\ g^{-1}$ of diesel particulate extract.[3,7] 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^{-3}, respectively. The concentrations of 1-NP associated with airborne particles (9.8−72.0 fmol m^{-3}) were comparable to those reported elsewhere in Japan.[32,33] 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 ($[M - H]^-$), together with the characteristic fragment ions, m/z 232 ($[M - H - NO]^-$) and 216 ($[M - H - NO_2]^-$) 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^{-1}, were 81% and 112% of the values certified by NIST, respectively.[34,35] The concentrations of DEP-associated 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP, 0.09−0.32 mg kg^{-1} for SRM 1650b and 0.01−0.03 mg kg^{-1} for SRM 1975, were much lower than that of 1-NP. The concentration ratios of ΣOHNPs (= 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 ΣOHNPs to 1-NP associated with airborne particles was 1.4; i.e., the value

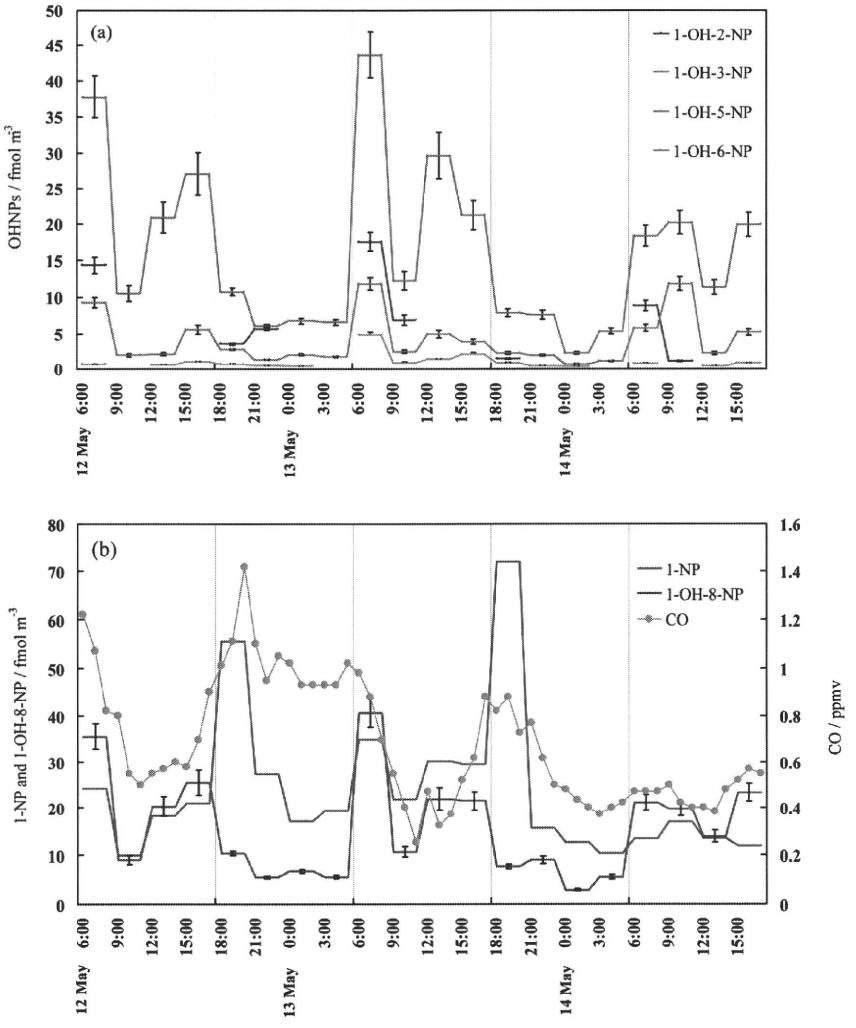

Figure 3. Diurnal concentrations of (a) OHNPs and (b) 1-NP and CO together with 1-OH-8-NP during May 12−14, 2003, at Sakai, Osaka. The concentration of each OHNP was corrected based on its degradation rate during high volume air sampling. Errors shown were calculated from one standard deviation derived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

of ΣOHNPs/1-NP in ambient airborne particles was higher than that in DEPs by factors of about 35 and 470. The low ΣOHNPs-/1-NP ratio in DEPs estimated as 0.04−0.2 by Schuetzle favorably compares with our results.[3] 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^{-3} (40 fmol m^{-3}) by Nishioka et al. were well consistent with our results.[36] Gibson et al. also quantitatively analyzed airborne particle-bound OHNPs, although they did not determine each OHNP isomer independently.[5] 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 ΣOHNPs/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.[5] 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.[11] 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.[11] 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.[37] 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.[13,20,23] 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,[11,38] with gaseous nitrating species such as NO_2, HNO_3, and N_2O_5. Several researchers reported the reactions of particle-associated PAHs such as pyrene and fluoranthene with the gaseous nitrating species.[39−41] The NPAH isomers formed from the heterogeneous reactions are the same as those formed from electrophilic nitration reactions involving NO_2^+ 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.[6] Electrophilic nitration of OHPy by NO_2^+ preferentially leads to 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP as in pyrene.[11] 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,[16,42] 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.[43] 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_3 radicals in the presence of NO_2.[44,45] 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.[2,44] As described above, OHPy is expected to be reactive toward both OH and NO_3 radicals. Both the OH and NO_3 radical-initiated reactions of OHPy in the gas phase could lead to OHNP formation by analogy with the similar reactions of phenol and cresols,[46] although vapor pressure of OHPy is significantly lower than those of the other species.[47] 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

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

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