表3　候補ペプチドの同定結果

peptide ID	m/z-L	Identified	*Possible candidates*
12-5	684.96	**Rat Serum Albumin**	
12-10	725.99	*not identified*	
12-20	598.78	**Major Urinary Protein Precursor**	
13-1	393.85		***Retinoic acid receptor RXR-beta***
			Receptor-interacting serine/threonine-protein kinase 5
13-10	509.9	**E3 ubiquitin-protein ligase rififylin**	

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分担研究報告書

分担研究課題：細胞レベルでのメタボロミクス技術の開発

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研究要旨

細胞レベルでの薬剤による毒性の予測を行うため、細胞試料を用いたNMRによるメタボノミクス技術の開発を目的とした。本年度は細胞への薬剤暴露時の網羅的遺伝子発現解析を実施するとともに、同じサンプルを用いたNMRによるメタボローム解析の検討を行った。遺伝子発現解析においてはそれぞれの薬剤暴露にレスポンスする遺伝子群を特定することが出来た。またメタボロミクス解析においてはPCA分析の結果、薬剤暴露のレスポンスを細胞が反映しているという結果を得ることが出来た。

研究目的

ヒトに投与して初めて起こる副作用を予測するため網羅的な遺伝子発現解析が注目されトキシコゲノミクスと呼ばれる研究が実施されてきた。しかし毒性の予測までには至っていないのが現状である。本研究では非侵襲試料を用いたメタボノミクス解析手法を確立し、トキシコゲノミクスで得られた網羅的な遺伝子発現情報を補完し、毒性予測につながる評価系開発を行うことを目的としている。そこで我々はよりスクリーニング研究に適していると考えられる培養細胞を用いたメタボノミクス解析技術の確立を目指す。本年度は細胞レベルでのメタボロミクス解析を行うため、ヒト肝･腎細胞およびヒト由来培養細胞を用いて、細胞を超音波破砕したものを試料とし、NMR にて解析する手法の条件検討を行った。

研究方法

＜細胞に関して＞

THP-1（急性単球性白血病）

毒性試験に非常によく用いられる細胞。

培地：RPMI1640 medium with 5% fetal calf serum.

継代方法：Dilution.

＜薬剤処理に関して＞

1:Control（DMSO 0.1%）
2:アセトアミノフェン（APAP） 200 μM
3:メトトレキサート（MTX） 120 μM
4:オメプラゾール（OPZ） 120 μM
5:チオアセタミド（TAA） 2000 μM
6:シスプラチン（CSP） 8 μM
7:ジエチルニトロソアミン（DEN）10000 μM

で細胞を処理し、24時間暴露した。24時間

後に DNA/RNA を抽出し、遺伝子発現解析に用いた。

＜GeneChip による遺伝子発現解析に関して＞

GeneChip による遺伝子発現解析には、各被験物質処理を行った細胞より調製した totalRNA 5 μg を使用した。

- 使用した RNA の濃度測定には NanoDrop ND-1000 (NanoDrop Technologies 社製) を用いてサンプル1μLを用いて測定した。
- 純度・品質の検定には Agilent 2100 バイオアナライザシステム (Agilent 社製) を用い RNA の断片化が起こっていないことを確認した上で研究に使用した。

GeneChip 測定に用いるサンプルの調製は Affymetrix 社のマニュアルに基づいて行い、作成したサンプルを用いて GeneChip HG-U133A Array を用いてハイブリ、ウォッシュ、測定を行った。

＜超音波破砕＞

細胞を酵素安定化バッファー中で氷冷し、マイクロソン微量超音波細胞破砕機 XL2000 にて細胞を超音波破砕した。

＜試料の前処理＞

Albumin Segregation Kit を用いて、培養液中の血清成分（特にアルブミン）を除去し、サンプルとした。

＜NMR 測定に関して＞

ブルカーバイオスピン社製 AVANCEⅡ８００US2 型により ^{1}H-NMR を測定した。

結果

本分担研究では、細胞レベルでのメタボロミクス技術の開発を目的としており、NMR を用いた高感度解析技術を採用する予定である。しかし、導入予定である 800MHzNMR 設備整備が遅れ、NMR 解析による基礎検討を実施した。本年度は細胞レベルでのメタボノミクス解析と遺伝子発現解析データとの比較解析を行った。細胞は THP-1 を用い、処理薬剤としてアセトアミノフェン、メトトレキサート、オメプラゾール、チオアセタミド、シスプラチン、ジエチルニトロソアミンを採用し、薬剤暴露 24 時間後の細胞より DNA/RNA を抽出するとともに、NMR 解析用の超音波破砕細胞液を作成し、アルブミン除去後、NMR にて解析を行った。

細胞試料の作成法として超音波による細胞破砕を用い、氷冷酵素安定化バッファー中で細胞を超音波破砕し、酵素活性 (G6PD (Glucose-6-phosphate dehydrogenase), LD (Lactate dehydrogenase), NP (Nucleoside phosphorylase)) を有した状態で細胞破砕を行った。

NMR 解析の前処理としてヒトアルブミンを除去するキット Albumin Segregation Kit を用い、多量に含まれるアルブミンの影響を除去し、より微量な成分まで検出できるように前処理を行った。

考察

細胞レベルでのメタボロミクス技術の開発を目的として、高感度かつ簡便解析が実施できる NMR を採用することとした。医薬基盤研究所では本年度 800MHz 高感度

LC-MS-NMR を設備整備したが、その整備が遅れ 1 月より実際の NMR 解析を実施した。

今回用いた THP-1 細胞は毒性研究に非常によく使用される細胞であり、その点ではこれまでに蓄積されたバックグラウンドデータとの比較が容易であると考えられた。

薬剤暴露した細胞の遺伝子発現解析データはコントロールと比較して変動した遺伝子群を抽出し（図１）、クラスター解析を行った（図２）。その結果オメプラゾールとシスプラチン暴露のデータが他の薬剤と異なるグループとなる結果であった。

また、細胞レベルでのメタボノミクス解析においては、NMR が非常に高感度であり、煩雑な前処理を必要としないことから、細胞を薬剤処理し、一定時間後超音波破砕した細胞試料をアルブミン除去後解析する方法を検討しており、実際に NMR 解析したところ、ノイズ等を気にすることなく解析が可能であるという結果を得ている。試料として用いる細胞破砕液に関してはいろいろな方法によって調製が可能であるが、簡便な方法であり、酵素活性も維持できるように、酵素安定化バッファー中での超音波破砕を実施した。本方法は発熱による酵素活性への永久尾が考えられるため、氷冷したバッファー中にて実施することにより、酵素活性を維持した状態での細胞破砕液調製を行うことができた。

NMR を用いた薬剤処理細胞のメタボローム データを PCA 解析すると、暴露した薬剤ごとにある程度分離できることがわかった。特に PCA 解析を行った時に、遺伝子発現のクラスター解析結果と薬剤の分類が一致していた（図３）。この結果から薬剤暴露した細胞の破砕液によるメタボローム解析が遺伝子発現解析によるトキシコゲノミクスデータを補完するのに有効な方法であるといえる。

今後はメタボロミクス解析手法の開発を継続し、網羅的遺伝子発現解析データであるトキシコゲノミクスデータの表現型を補完する非常に有効な手法として技術確立を継続する予定である。

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知的所有権の取得状況

なし

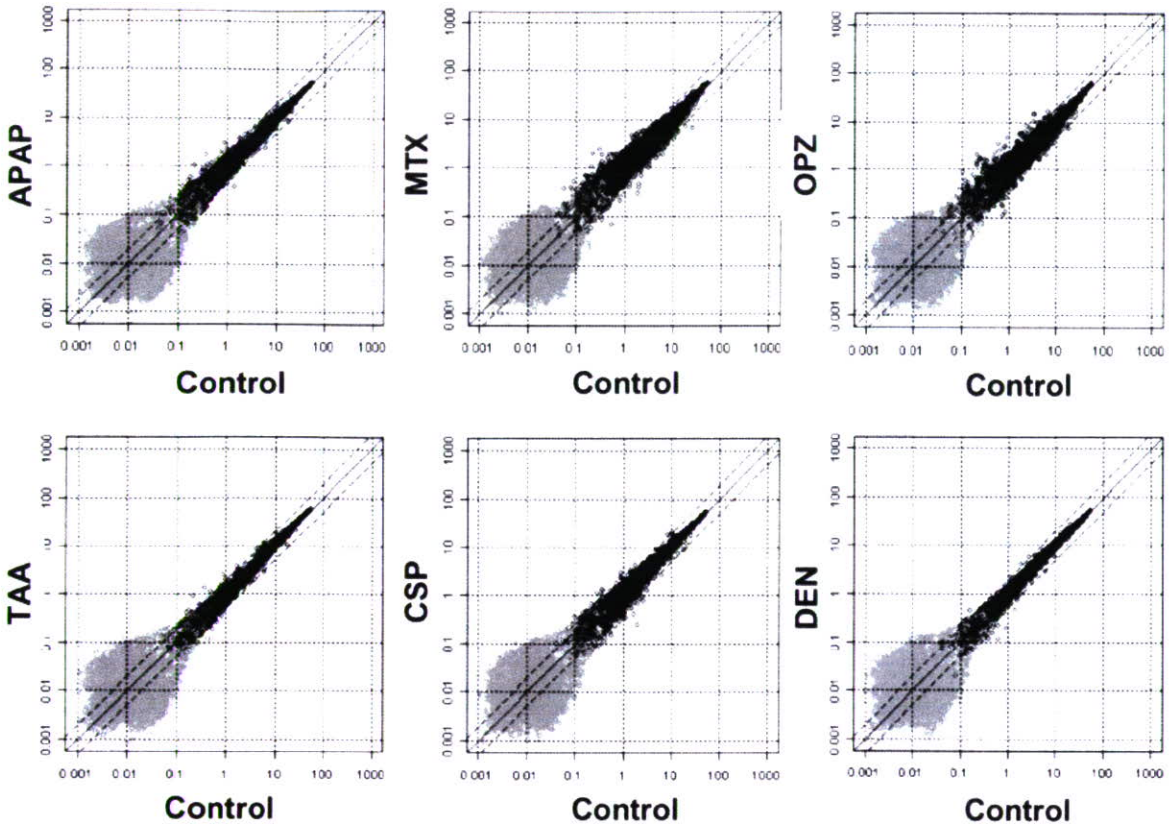

図１　遺伝子発現解析結果（薬剤処理とControlのスキャッタープロット）

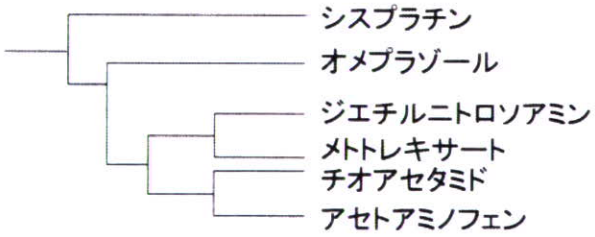

図２　薬剤処理による変動遺伝子を用いたクラスター解析

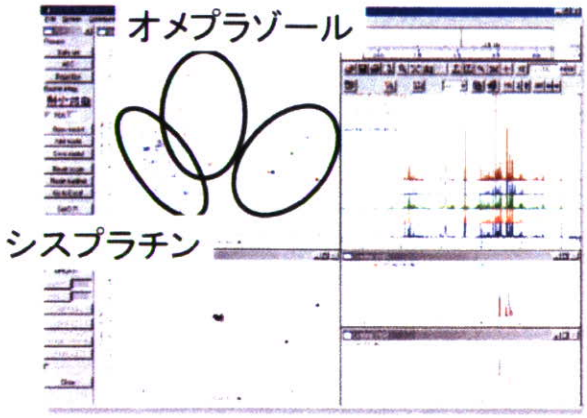

図３　薬剤処理細胞破砕液のＰＣＡ解析結果

別紙４

研究成果の刊行に関する一覧表

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Present State of New Chiral Drug Development and Review in Japan

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The current situation of chiral drug development in Japan was investigated. The trend in the Japanese pharmaceutical development is increasingly moving towards the development of single isomers rather than racemates. The development of single-enantiomer drugs was made possible by the current technologies of asymmetric synthesis and chiral separation, and encouraged by the guidelines on the development of chiral drugs worldwide. Japan has not issued specific guidelines on the development of chiral drugs, however, the chiral drug development approached in Japan were essentially consistent with the approaches recommended by the U.S.A. and EU guidelines.

Key words —— chiral drug, single-enantiomer drug, racemic drug, pharmaceutical development, guideline

INTRODUCTION

Pharmaceuticals with an asymmetric carbon (chiral center) are often referred to as chiral drugs. Chiral drugs were mainly presented as the racemate, which is a mixture of equal amounts of left- and right-handed enantiomers. Two enantiomers in a racemate show completely identical physical and chemical properties when they are in an achiral environment. However, in a chiral environment such as *in vivo*, they demonstrate different chemical, biochemical, and pharmacologic behaviors. In principle, therefore, enantiomers in a racemic drug should be treated as two different compounds.

Although single-enantiomer drugs have been thought to be preferable to racemic drugs, most chiral drugs were developed as racemates due to the lack of technologies that produce single-enantiomers until recently. Current technologies of asymmetric synthesis and chiral separation made it possible for pharmaceutical companies to develop single-enantiomer drugs. Lately, many single-enantiomer drugs have been approved and marketed broadly. The Japanese government approved single-enantiomer drugs four times as much as racemic drugs in early 2000s, although there was not significant difference in the number of approved drugs between racemic and single-enantiomer drugs in early 1990s (Fig. 1). The trends in world development and approval of chiral drugs were similar; the worldwide market share of single-enantiomer drugs increased from 27% in 1996 to 39% in 2002.[1–3]

Pharmaceutical companies cannot market new drugs in Japan and in other regions until approved by the regulatory authorities, and they have to submit technical documents for new drug applications. The regulatory authorities evaluate the content of the technical documents to assure quality, efficacy and safety. In order to assure drug quality[4] in Japan, specifications and critical manufacturing processes are needed to be written in legally binding approval documentation. In contrast, almost all of subjects described in technical documents are considered as legally binding matters in other countries. As a result, approval matters on quality-related issues are often different between Japan and other countries.

We considered it important to research what kinds of data are to be collected during the development of chiral drugs and how the regulatory authorities evaluate these data. Additionally it is valuable to realize what kinds of issues are dealt with as legally binding matters. The findings can provide common understanding between pharmaceuti-

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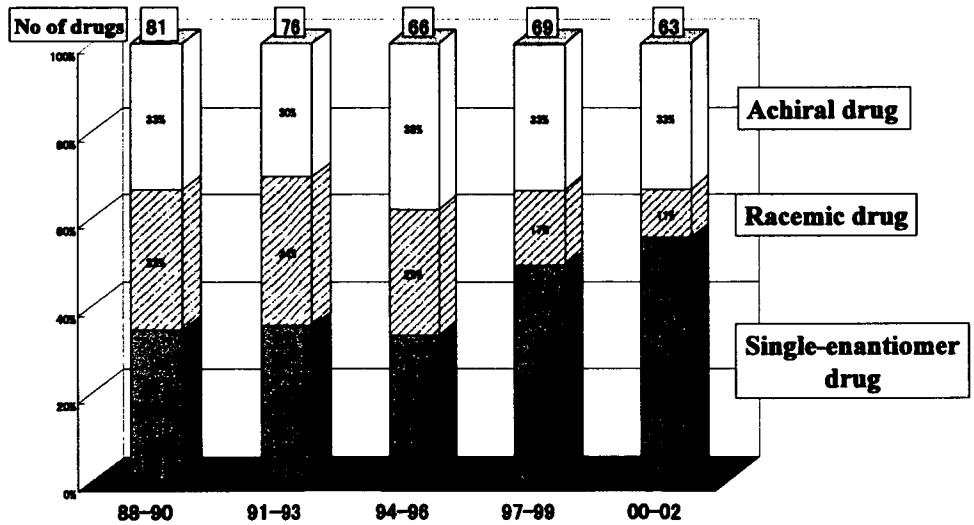

Fig. 1. Number of Drugs Approved in Japan during 1988–2002

The new active ingredients, which are chemical substances and were approved in Japan between 1988 and 2002, were classified according to steric structures.

cal companies and the regulatory authorities, and facilitate development of chiral drugs.

In this article, we analyzed data on manufacturing, quality, pharmacology, toxicology, and pharmacokinetics of single-enantiomer and racemic drugs that were approved by the Japanese government, and discussed the current state of development and approval of chiral drugs.

MATERIALS AND METHODS

The drugs that were approved from January 2001 to July 2003 were surveyed. The information sources were the data summaries [Module 2 of Common Technical Document (CTD) in the present system] that were submitted by the applicants for New Drug Application (NDA) and the approval documentations that were described specifications and test methods of drug substances. This information, especially quality parts, is not completely publicly available, although Module 2 of CTD and review reports available on the Internet.[5,6] Therefore, we did not disclose the individual substances' name.

In this article, the term of 'chiral drug' contains both a single-enantiomer and a racemic drug.

RESULTS

Classification of Approved New Chiral Drugs by Stereochemistry (Fig. 2)

There were 76 new active ingredients that were approved between January 2001 and July 2003 excluding biologics, antiseptics for medical devices and *in vivo* diagnostics.

We classified them into achiral drugs, racemic drugs and single-enantiomer drugs with one-chiral center or multi-chiral centers. The 76 new substances consisted of the 29 achiral drugs (black portion, 39%), the 23 single enantiomer drugs with multi-chiral centers drugs (strip portion, 30%), the 14 single enantiomers with one-chiral center (gray portion, 18%), and the 10 racemic drugs (white portion, 13%). The total number of single enantiomer was 37.

We investigated further details of the 37 single-enantiomer drugs and the 10 racemic drugs as shown in the following sections.

Single-enantiomer Drug Substances

Manufacturing Routes (Fig. 3): The 29 single-enantiomer drugs out of the 37 investigated drugs were produced from single enantiomeric starting materials (gray portion, 78%). In this research, the starting materials indicate starting compounds by manufacturing method in approval documentation and in good manufacturing practices (GMP) compliance.

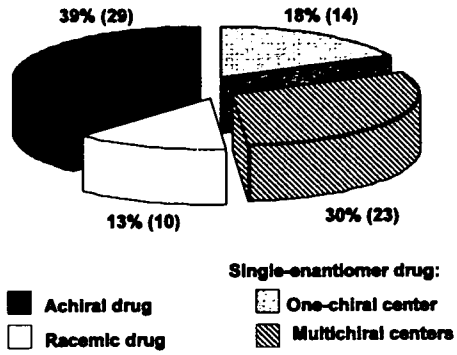

Fig. 2. Classification of New Chemical Drugs by Stereochemistry

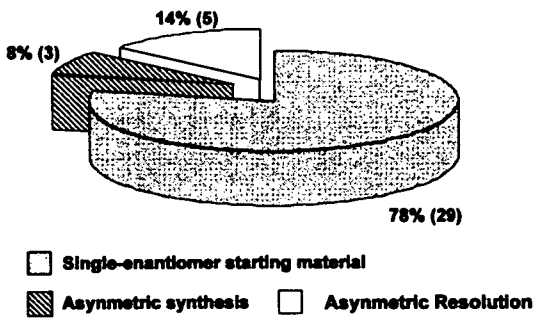

Fig. 3. Manufacturing Routes to Single-enantiomer Drug Substances

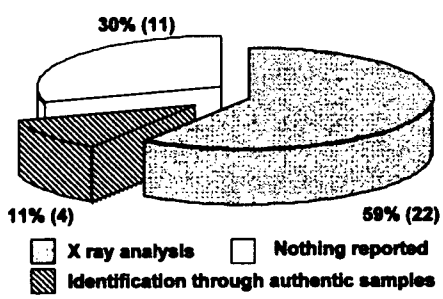

Fig. 4. Stereochemical Characterizations on Chirality of Single-enantiomer Drug Substances

Asymmetric synthesis was used for formation of single-enantiomers for the three single-enantiomers (stripe portion, 8%).

The five substances were isolated by asymmetric resolution (white portion, 14%). The four substances out of the five were purified by crystallization. The other one was purified by chromatographic resolution.

Stereochemical Characterizations on Chirality (Fig. 4): The stereochemical structures of 22 single-enantiomers were determined by X-ray crystal structure analysis (gray portion, 59%), and 4 single-enantiomers were confirmed by identification through the authentic samples (stripe portion, 11%). No information was reported on characterization of chirality for the remaining 11 single-enantiomers (white portion, 30%).

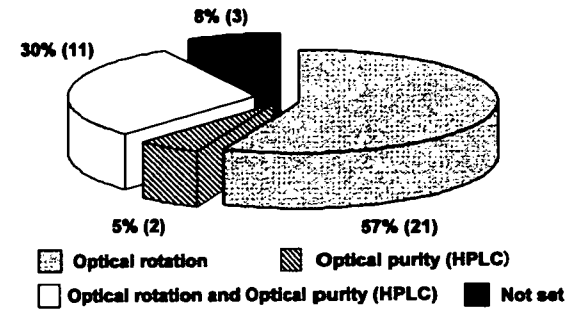

Fig. 5. Specifications for Assuring Chirality of Single-enantiomer Drug Substances

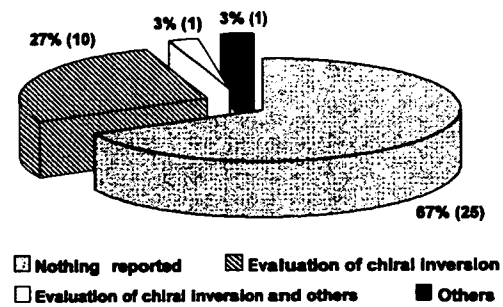

Fig. 6. Pharmacokinetic Studies on Chirality of Single-enantiomer Drug Substances

Specifications for Assuring Chirality (Fig. 5): Optical rotation was adopted as specifications for the 21 single-enantiomers (gray portion, 57%). The chromatographic methods for optical purity determination were chosen for the two enantiomers (strip position, 5%). Both optical rotation and optical purity were adopted as specifications for the 11 enantiomers (white portion, 30%).

No specification for assuring chirality was set for the three enantiomers (black portion, 8%).

Pharmacokinetic Studies on Chirality (Fig. 6): Pharmacokinetic studies were conducted for all of the single-enantiomer drugs, and some sort of pharmacokinetic evaluation relating to chirality was reported for the 12 single-enantiomers. Among them, chiral inversion was evaluated for the 10 single-enantiomers (strip portion, 27%). One out of the 10 single enantiomers indicated chiral inversion on

mouse, although the other 9 single enantiomers did not show any chiral inversion.

One of the other two single enantiomers was proved to generate an enantiomer-specific metabolite (black portion, 3%); and the other one, which did not show any chiral inversions, was additionally investigated on the chiral inversion in metabolite formation (white portion, 3%).

No pharmacokinetic study on chirality was reported in the data summaries of the 25 single-enantiomers (gray portion, 67%).

Racemic Drug Substances

Stereochemical Characterization (Fig. 7): All of the 10 racemic drugs were confirmed to be a racemic substance by some methods. Optical rotation was reported for all of the 10 racemates. Chiral HPLC analysis and X-ray analysis was reported for the nine and the four racemates, respectively. For the four racemate, both chiral HPLC analysis and X-ray analysis were performed.

Pharmacology of Individual Isomers (Fig. 8): Pharmacological activity of each enantiomer was investigated for all of the 10 racemic drugs. Little difference was observed in pharmacological activities of both enantiomers in the five racemates (stripe portion, 50%). For the four racemates, each enantiomer in the racemate indicated different pharmacological potency (gray portion, 40%). For the three of the four racemates, the enantiomers that had greater pharmacological potency were more toxic. The mechanisms of toxicities were same as those of pharmacological activities. In the other racemate, the one enantiomer indicated equivalent toxic potency to the racemate.

Different results were observed between both enantiomers depending on assay systems for the one racemate (white portion, 10%). *In vivo* assay the component isomers in this racemate did not show much difference.

Single-dose Toxicity of Individual Isomers (Fig. 9): Individual single-dose toxicity was reported for 7 drugs out of the 10 racemic drugs. For the three racemates, both enantiomers indicated different single-dose toxicity (gray portion, 30%), and the enantiomer that had higher pharmacological activity was more toxic. Therefore, the development of single-enantiomer drugs was not necessarily beneficial in terms of both safety and efficacy, and the applicants developed the drugs as racemates.

For the four racemates, the individual enantiomers were not different at single-dose toxicity

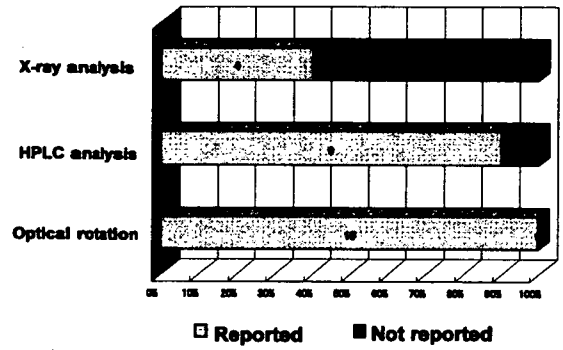

Fig. 7. Stereochemical Characterizations on Chirality of Racemic Drug Substances

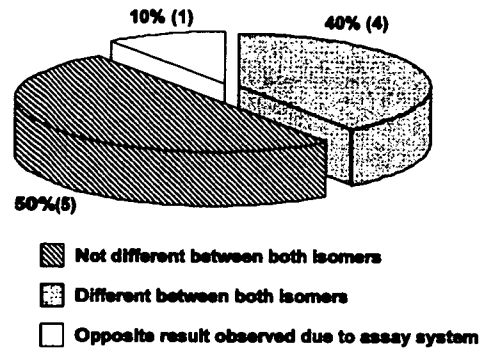

Fig. 8. Pharmacology of Individual Isomers in Racemic Drugs

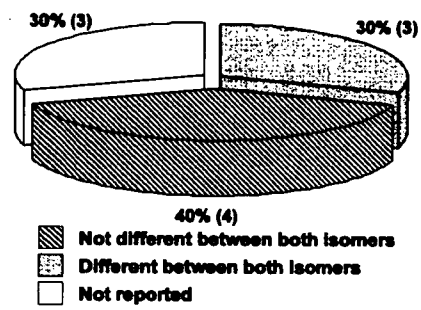

Fig. 9. Single-dose Toxicity of Individual Isomers in Racemic Drugs

(stripe portion, 40%).

Both enantiomers in the three racemates, which had no toxic information of individual isomers, indicated similar pharmacological activities.

Pharmacokinetic Study of Individual Isomers (Fig. 10): Pharmacokinetic evaluations of individual enantiomers were performed for nine racemates using experimental animals and/or human including human tissue-derived materials (Fig. 10). Chiral in-

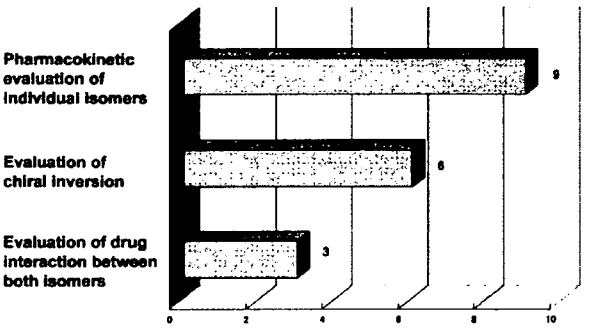

Fig. 10. Classification of Pharmacokinetic Studies on Chirality of Racemic Drugs

version was evaluated for the six racemates, all of which were not observed any chiral inversion. Drug interaction between both enantiomers of the three racemic drugs was investigated. In the other one racemic drug, no pharmacokinetic study relating to chirality was reported.

Pharmacokinetic profiles of enantiomers were investigated for the eight racemic drugs using experimental animals. The four racemic drugs indicated that the individual enantiomers have different profiles. However, there was no difference between both isomers in the rest of four racemates.

Pharmacokinetics was studied using human (healthy volunteers, human tissue-derived materials) for the seven racemates. Pharmacokinetic profiles were different between both enantiomers of the three racemic drugs. The enantiomers of the racemic drug demonstrated different results depending on experimental subjects.

For the four racemates of which each enantiomer indicated different pharmacological potency, both pharmacokinetic profiles of individual enantiomers and chiral inversion were investigated. Drug interaction between both enantiomers was evaluated in the two racemates out of the four.

DISCUSSIONS

Development of single-enantiomer drugs was made possible by the introduction of asymmetric synthesis and chiral separation technologies. In addition, the publication of several guidelines dealing with chiral drugs[7–10] encouraged the development of single-enantiomer drugs for pharmaceutical manufacturers. The following problems of racemic drugs were parts of the reason for making those guidelines:[7–9] One member of an enantiomeric pair might be pharmacologically active and the other inactive; Enantiomers might have different concentration-response relationships for some property; Enantiomers might have completely different activities; Toxicity of a racemic drug might be linked to one member of enantiomeric pairs; Enantiomers might have different pharmacokinetic behavior.

North America[7,8] and Europe[9] have their original guidelines on chirality that describe manufacturing, quality, pharmacology, toxicology, pharmacokinetics and so on. Additionally, quality of chiral drugs was stipulated by a guideline of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceutical Human Use (ICH). The guideline, entitled "Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances"[10] and encoded as Q6A, recommends applicants, in case of development of single-enantiomer drugs, to consider the other enantiomer as an impurity and to set the identity tests capable of distinguishing both enantiomers and the racemic mixture.

Japan has not issued specific guidelines on the development of chiral drugs. ICH-Q6A was implemented officially in Japan on May 1, 2001 and came into effect on July 1, 2001.[11] There was a transitional period from the previous guideline until July 1, 2003. Although the drugs surveyed in this paper are not necessarily objects of ICH-Q6A, applicants could prepare to follow ICH-Q6A since it reached Step 4 in October 1999. Additionally, the Japanese regulatory authorities indicated that where the active ingredient is an optical isomer, a method of discriminating between enantiomers should be investigated and the ratio of enantiomers determined.[12–15] Except for the quality point of view, 'Guidelines on Non-clinical Pharmacokinetic Studies'[16] says that when the investigational substance is a racemate, sponsors should monitor the enantiomers individually to determine the pharmacokinetic profiles. The developments of chiral drugs in Japan must be affected by U.S.A. and/or EU guidelines because pharmaceutical developments parallel in worldwide.

The above guidelines do not prescribe a selection strategy to develop as a single enantiomer or a racemate. However, applicants would be expected to provide a scientifically based justification when a racemic drug had been developed instead of a single-enantiomer drug.

Single-enantiomer Drug Substances

For the 11 single-enantiomers, the stereochemical characterization was not reported (Fig. 4). It does not necessarily mean that the Japanese authorities did not receive any information, but it means that no stereochemical characterization was described in the data summaries. In Japan, reviewers use data summaries as a primary assessment document and technical reports as an additional tool, because significant issues are described in data summaries in the Japanese review system. On the other hand, reviewers directly assess individual technical reports on chemistry, manufacturing and control (CMC) in U.S.A.

The stereochemical characterization was reported for all the substances which were produced by asymmetric synthesis or asymmetric resolution, and hence we supposed that the stereochemical characterizations of the 11 single-enantiomers were regarded as less significant for the following reason: Those 11 substances were synthesized from single-enantiomeric starting materials containing multi-chiral centers such as sugars and steroids; Synthetic procedures of those substances were regarded as ensuring their stereochemistry.

ICH-Q6A guideline says that the identity tests should be capable of distinguishing both enantiomers and the racemic mixture for a drug substance developed as a single enantiomer. However, the specification for assuring chirality was not adopted for the three single-enantiomer drugs. One of them had one-chiral center, and the others had multi-chiral centers. Although the reason for rejected specifications is not clear from the data summaries, it might result from the fact that the NDA of three drugs were submitted during the transitional period for implementing Q6A, or those three substances might be regarded as retaining their starting material chirality, or the specification for assuring chirality might be considered to be meaningless.

Also, no pharmacokinetic study on chirality was reported in the data summaries of the 25 (67%) single enantiomers (Fig. 6), 19 of which had multi-chiral centers. In contrast, the chiral inversion was reported for the four single enantiomers with multi-chiral centers, however, less or equal two chiral centers were investigated in those enantiomers. For the single enantiomer with multi-chiral centers, their chiral inversion and isomer-specific metabolism tended to be considered to be complicated and unimportant.

Racemic Drug Substances

Based on the guidelines, we reassessed the justifications for developing the 10 racemates rather than a single enantiomer.

The pharmacologic activity and the pharmacokinetic profile of the individual enantiomers should be characterized, because rapid interconversion *in vivo* was not observed for all of the 10 racemic drugs. Although the principal pharmacological activity was characterized for the individual isomers of the 10 racemic drugs, the pharmacokinetic profiles of the individual enantiomers were not reported for the one. It might be one of the reasons for not reporting that this racemic drug has been a common therapeutic agent for long time in worldwide.

According to the guidelines, it is ordinarily sufficient to carry out toxicity studies on the racemate. The toxicity study of each isomer was not reported for the three racemic drugs, because both enantiomers of these three racemates indicated similar pharmacological activities.

Relating to stereochemical characterization, it is recommended to perform chromatographic tests in addition to optical rotatory tests for mixtures of optical isomers. The chiral HPLC analysis was not performed for the one racemic drug. This racemic drug has been a common therapeutic agent for long time in worldwide, and the Japanese authorities did not require additional stereochemical characterization.

In conclusion, the trend in the Japanese pharmaceutical development is increasingly moving toward the development of single isomers rather than racemates. The chiral development approaches approved in Japan were essentially consistent with the approaches recommended by the guidelines. The racemic drugs, which shared only 13% in the new chemical drug substances, had some rationale to be developed as racemates. Decline of racemic drugs development may continue in Japan as well as worldwide, because some studies need to be carried out with not only a racemic mixture but its component enantiomers.

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nor should be inferred.

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Communication

Effect of Methyl Substitution on the Antioxidative Property and Genotoxicity of Resveratrol

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Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a natural phytoalexin with various biological activities including inhibition of lipid peroxidation and free radical scavenging properties. In addition to its beneficial effects, resveratrol also has significant genotoxicity that leads to a high frequency of chromosome aberration together with micronucleus and sister chromatid exchanges. To enhance the radical scavenging activities and to reduce the genotoxicity of resveratrol, we designed 4′-methyl resveratrol analogues where a methyl group was introduced at the ortho position relative to the 4′-hydroxy group, which is responsible for both antioxidative activities and genotoxicity of resveratrol. These synthesized methyl analogues of resveratrol showed increased antioxidative activities against galvinoxyl radical as an oxyl radical species. Furthermore, the methyl analogues also surprisingly showed reduced in vitro genotoxicities, suggesting that methyl substitution may improve resveratrol efficacy.

Introduction

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a natural phytoalexin present in grapes and wine that has been shown to play an essential role in the prevention of several human pathological processes including inflammation (*1*), atherosclerosis (*2*), and carcinogenesis (*3*). The cancer preventive activity of resveratrol is linked to its ability to eliminate free radicals and to reduce oxidative and mutagenic stress. Lipid peroxidation is one of the basic mechanisms of cell and tissue damage leading to various diseases. So far, the protective effect toward lipid peroxidation has proved to be a typical antioxidative event of resveratrol (*4*). It has been demonstrated that resveratrol suppresses lipid peroxidation by both scavenging of free radicals and chelation of copper (*5*). Among three hydroxyl groups in resveratrol, the 4′-hydroxyl group is essential for radical scavenging activities (*6*). Besides, the 4′-hydroxyl group has been primarily responsible for the copper binding property (*7*). In addition to its beneficial effects, resveratrol is also reported to be genotoxic, inducing a high frequency of chromosomal aberrations (CA), micronucleus, and sister chromatid exchanges (SCE) in vitro (*8*). Structure–activity relationship studies of resveratrol analogues revealed that the 4′-hydroxyl group, besides being essential for antioxidative activity, is also responsible for the in vitro cytogenetic activity of resveratrol (*7*, *9*, *10*). In this regard, our challenge is to create novel resveratrol analogues that not only exert enhanced antioxidative abilities but also have reduced in vitro genotoxicity. Such analogues could lead to the development of new drugs against various diseases, particularly those related to oxidative stress. In our attempt to design new resveratrol analogues, we focused on the methyl groups of the tocopherol due to their proven antioxidative effects on the aromatic ring (*11*). In particular, methyl groups at the ortho position to the hydroxyl group contribute to delocalization of the unpaired electron of the corresponding phenoxyl radical, which is generated in the reaction with radical species, due to hyperconjugation. In this study, we describe resveratrol analogues where methyl groups are introduced into the ortho position of the 4′-hydroxyl group. Their designs are relatively simple, but in comparison

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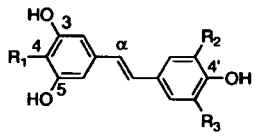
Scheme 1. Structure of Resveratrol and Methyl Derivatives



	R_1	R_2	R_3
resveratrol	H	H	H
1	H	CH_3	H
2	H	CH_3	CH_3
4-methylresveratrol	CH_3	H	H
3	CH_3	CH_3	H
4	CH_3	CH_3	CH_3

with resveratrol, their antioxidative abilities are significantly increased, and surprisingly, in vitro genotoxicities are also decreased.

Experimental Procedures

General. The reagents and solvents used were of commercial origin (Wako Chemicals, Tokyo Chemical Industry, Sigma, and Aldrich) and were employed without further purification. The ^1H NMR spectra and ^{13}C NMR spectra were recorded with a Varian AS 400 Mercury spectrometer. Chemical shifts are expressed in ppm downfield shift from TMS (δ scale). Low- and high-resolution mass spectra were obtained on a JEOL MS700 mass spectrometer. The progress of all reactions was monitored by thin-layer chromatography on silica gel 60 F_{254} (0.25 mm, Merck). Column chromatography was performed on silica gel 60 (0.063–0.200 mm, Merck). The purity of all synthetic compounds was approximately >98% (based on ^1H NMR spectra).

Diethyl 3,5-bis(benzyloxy)benzylphosphonate (5a). Triethyl phosphite (2.49 g, 15mmol) was added to a mixture of 3-benzyloxy-4-methylbenzylbromide (**11a**) (3.83 g, 10 mmol) and tetrabutylammonium iodide (73 mg, 0.2 mmol), and the resulting mixture was heated for 8 h. Excess triethyl phosphite was removed by heating for 3 h at 60 °C under vacuum to yield 4.32 g of **5a** (98% yield) as a light yellow oil. ^1H NMR (400 MHz, DMSO-d_6): δ 1.21 (t, 6H), 3.90 (q, 4H), 5.09 (s, 4H), 6.55 (d, 2H), 6.57 (s, 1H), 7.4 (m, 12H). MS (EI *m/z*) 440 [M^+].

Methyl 3,5-dihydroxy-4-methylbenzoate (8). To a solution of 3,5-dihydroxy-4-methylbenzoic acid (10.08 g, 60 mmol) in methanol (150 mL), sulfuric acid (3.0 mL) was added. The resulting mixture was stirred for 18 h and poured into ice water (200 mL), and then, the product was extracted with ethyl acetate (3 × 300 mL). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate = 1:1) to afford 9.40 g of **8** (86% yield) as a colorless oil. ^1H NMR (400 MHz, DMSO-d_6): δ 1.96 (3H, s), 3.75 (3H, s), 6.91 (2H, s), 9.28 (2H, br). MS (EI *m/z*) 182 [M^+].

Methyl 3,5-bis(benzyloxy)-4-methylbenzoate (9). To a well-stirred solution of **8** (6.64 g, 36.5 mmol) in DMF (100 mL), potassium carbonate (23.49 g, 170 mmol) was added under argon. After 1 h, benzyl bromide (15.6 g, 2.5 equiv, 91.3 mmol) was added dropwise over a period of 30 min and the reaction was allowed to proceed for 16 h. The reaction mixture was poured into ice water (200 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The organic fractions were combined and washed with water and brine and then dried over anhydrous Na_2SO_4. The residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate = 2:1) to afford 10.44 g of **9** (79% yield) as a white powder. ^1H NMR (400 MHz, DMSO-d_6): δ 2.15 (3H, s), 3.82 (3H, s), 5.19 (4H, s), 7.27 (2H, s), 7.40 (10H, m). MS (EI *m/z*) 362 [M^+].

3,5-Bis(benzyloxy)-4-methylbenzyl alcohol (10). A solution of **9** (7.25 g, 20.0 mmol) in anhydrous diethyl ether (50 mL) was added to a cold suspension of lithium aluminum hydride (1.14 g, 30 mmol) in anhydrous diethyl ether (100 mL) over the period of 30 min. The reaction mixture was stirred for 6 h. Excess lithium aluminum hydride was decomposed by successive dropwise addition of 2-propanol and 10% potassium hydroxide. The organic layer was then passed through a Celite filter pad, and the filtrate was dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum to afford 6.09 g of **10** (91% yield) as a white powder. ^1H NMR (400 MHz, DMSO-d_6): δ 2.06 (3H, s), 4.43 (2H, d, J = 5.6 Hz), 5.08 (4H, s), 5.15 (1H, t, J = 5.6 Hz), 6.69 (2H, s), 7.40 (10H, m). MS (EI *m/z*) 334 [M^+].

3,5-Bis(benzyloxy)-4-methylbenzyl bromide (11b). Phosphorus tribromide (2.71 g, 10 mmol) was added to a stirred solution of **10** (3.34 g, 10 mmol) in dry CH_2Cl_2 (50 mL) at 0 °C under argon. The stirring was continued for 2 h at 0 °C and at room temperature for 2 h. The reaction mixture was poured onto ice water and extracted with diethyl ether (3 × 100 mL). The ether layers were combined, washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum, and the residue was purified by silica gel column chromatography (*n*-hexane:CH_2Cl_2 = 1:1) to afford 10.44 g of **11b** (3.62 g, 91% yield) as white needles. ^1H NMR (400 MHz, DMSO-d_6): δ 2.06 (3H, s), 4.64 (2H, s), 5.09 (4H, s), 6.85 (2H, s), 7.40 (10H, m). MS (EI *m/z*) 397 [M^+].

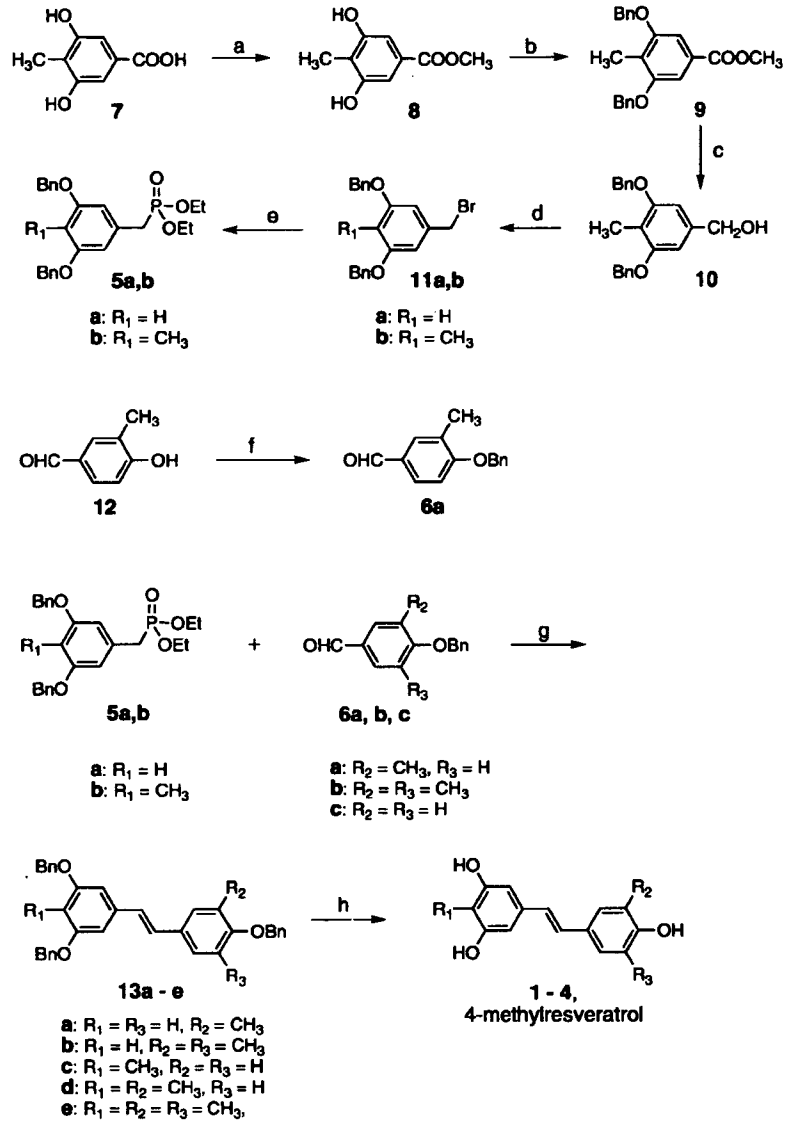
Diethyl 3,5-dibenzyloxybenzyl phosphonate (5b). Triethyl phosphite (3.0 g, 18 mmol) was added to **11b** (4.77 g, 12 mmol) containing a catalytic amount of tetrabutylammonium iodide, and the resulting mixture was heated at 120 °C for 12 h. Excess triethyl phosphite was removed by evaporater at 50 °C to afford **5b** (5.18 g, 95% yield) as a light yellow oil. ^1H NMR (400 MHz, DMSO-d_6): δ 1.24 (6H, t, J = 7.2 Hz), 2.05 (3H, s), 3.86 (4H, quint, J = 7.2 Hz), 5.06 (4H, s), 6.66 (2H, s), 7.40 (12H, m). MS (EI *m/z*) 454 [M^+].

4-Benzyloxy-3-methylbenzaldehyde (6a). To a well-stirred solution of 4-hydroxy-3-methylbenzaldehyde (**12**) (3.40 g, 25 mmol) in DMF (100 mL), potassium carbonate (11.05 g, 80 mmol) was added under argon. After 1 h, benzyl bromide (10.68 g, 2.5 equiv, 62.5 mmol) was added dropwise over a period of 30 min and the reaction was allowed to proceed for 14 h. The reaction mixture was poured on ice water (200 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The organic fractions were combined and washed with water and brine and dried over anhydrous Na_2SO_4. The residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate, 2:1) to afford 4.98 g of **6a** (88% yield) as a colorless oil. ^1H NMR (400 MHz, DMSO-d_6): δ 2.15 (3H, s), 3.82 (3H, s), 5.19 (4H, s), 7.27 (2H, s), 7.40 (10H, m). MS (EI *m/z*) 226 [M^+].

General Procedure for the Preparation of Stilbenes (13). Sodium hydride (0.2 g, 4 mmol) was added to a well-stirred suspension of the phosphate esters **5a,b** (2 mmol) in dry THF(10 mL) at −8 °C under argon. After 30 min, the aldehydes **6a**−**c** (2 mmol) in dry THF (15 mL) were added dropwise, and the reaction mixture was allowed to stir at room temperature for 16 h. The mixture was then cooled to 0 °C, and the excess sodium hydride was quenched with water (20 mL). The reaction mixture was then poured on ice, followed by addition of 2 M HCl (5 mL), and the products were extracted with ethyl acetate (2 × 100 mL). The organic layers were combined and were washed with brine. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The purification of the crude product was performed by silica gel chromatography (*n*-hexane: CH_2Cl_2 = 2:1).

(*E*)-1-(Benzyloxy)-4-[3,5-bis(benzyloxy)styryl]-2-methylbenzene (13a). From **5a** and **6a**. Yield, 62.2%. ^1H NMR (400 MHz, DMSO-d_6): δ 2.21 (3H, s), 5.10 (4H, s), 5.13 (2H, s), 6.54 (1H, dd, J = 2.0, 2.0 Hz), 6.82 (2H, d, J = 2.0 Hz), 6.98 (1H, d, J = 16.0 Hz), 7.01 (1H, d, J = 8.4 Hz), 7.16 (1H, d, J = 16.0 Hz), 7.40 (17H, m). MS (EI *m/z*) 512 [M^+].

Scheme 2. Synthesis of Methyl Derivatives of Resveratrol[a]



[a] (a) H_2SO_4, MeOH, 25 °C (18 h). (b) $C_6H_6CH_2Br$, K_2CO_3, DMF, 25 °C (16 h). (c) $LiAlH_4$, Et_2O, 25 °C (6 h). (d) PBr_3, CH_2Cl_2, 25 °C (2 h). (e) $P(OEt)_3$, $(n\text{-}Bu)_4N^+I^-$, 120 °C (12 h). (f) $C_6H_6CH_2Br$, K_2CO_3, DMF, 25 °C (14 h). (g) NaH, THF, 25 °C (16 h). (h) $AlCl_3$, $(CH_3)_2NC_6H_6$, CH_2Cl_2, 0 °C (16 h).

(*E*)-2-(Benzyloxy)-5-[3,5-bis(benzyloxy)styryl]-1,3-dimethylbenzene (13b). From **5a** and 4-benzyloxy-3,5-dimethylbenzaldehyde (**6b**). Yield, 78.9%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (6H, s), 4.80 (2H, s), 5.12 (4H, s), 6.57 (1H, dd, J = 2.0, 2.0 Hz), 6.85 (2H, d, J = 2.0 Hz), 7.04 (1H, d, J = 16.4 Hz), 7.17 (1H, d, J = 16.4 Hz), 7.28 (2H, s), 7.40 (15H, m). MS (EI *m/z*) 526 [M^+].

(*E*)-1,3-Bis(benzyloxy)-5-[4-(benzyloxy)styryl]-2-methylbenzene (13c). From **5b** and 4-(benzyloxy)benzaldehyde (**6c**). Yield, 75.7%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.08 (3H, s), 5.11 (2H, s), 5.15 (4H, s), 6.95 (2H, s), 7.01 (1H, d, J = 16.0 Hz), 7.19 (1H, d, J = 16.0 Hz), 7.40 (19H, m). MS (EI *m/z*) 512 [M^+].

(*E*)-1,3-Bis(benzyloxy)-5-[4-(benzyloxy)-3-methylstyryl]-2-methylbenzene (13d). From **5b** and **6a**. Yield, 62.4%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07 (3H, s), 2.21 (3H, s), 5.13 (2H, s), 5.15 (4H, s), 6.94 (2H, s), 7.01 (1H, d, J = 16.0 Hz), 7.15 (1H, d, J = 16.0 Hz), 7.40 (18H, m). MS (EI *m/z*) 526 [M^+].

(*E*)-1,3-Bis(benzyloxy)-5-[4-(benzyloxy)-3,5-dimethylstyryl]-2-methylbenzene (13e). From **5b** and **6b**. Yield, 67.9%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.09 (3H, s), 2.26 (6H, s), 4.81 (2H, s), 5.17 (4H, s), 6.97 (2H, s), 7.06 (1H, d, J = 16.0 Hz), 7.17 (1H, d, J = 16.0 Hz), 7.28 (2H, s), 7.40 (15H, m). MS (EI *m/z*) 540 [M^+].

General Procedure for the Cleavage of the Benxyloxy Groups to Afford Methyl Resveratrols (1–4 and 4-Methylresveratrol). To a well-stirred solution of stilbene **13** (1 mmol) in dry CH_2Cl_2 (10 mL), *N*,*N*-dimethylaniline (3 mmol) was added under argon atmosphere at 0 °C. After 5 min, anhydrous $AlCl_3$ (4 mmol) was added to the reaction mixture. After 16 h, the reaction mixture was quenched with water at 0 °C. The reaction mixture was poured into a 1.0 M solution of HCl (20 mL). The resulting mixture was extracted with ethyl acetate (2 × 100 mL), and combined extracts were washed with brine (2 × 50 mL). The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The purification of the crude product was done by thin-layer chromatography under argon atmosphere (silica gel 60, 2 mm coated silica plate, CH_2Cl_2:MeOH = 10:1).

(*E*)-5-(4-Hydroxy-3-methylstyryl)benzene-1,3-diol (1). From **13a**. Yield, 50.4%; mp 217–219 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.12 (3H, s), 6.09 (1H, dd, J = 2.0, 2.0 Hz), 6.35 (2H, d, J = 2.0 Hz), 6.74 (1H, d, J = 8.0 Hz), 6.78 (1H, d, J = 16.4 Hz), 6.87 (1H, d, J = 16.4 Hz), 7.18 (1H, dd, J = 2.0, 8.0 Hz), 7.29 (1H, d, J = 2.0 Hz), 9.18 (2H, s), 9.44 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.2, 153.1, 140.3, 131.5, 128.6,