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別紙１

厚生労働科学研究費補助金

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新規磁性薬剤化合物の画像診断への応用に関する研究

平成１９年度　総括研究報告書

主任研究者　石川義弘

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厚生労働科学研究費補助金（医療機器開発推進研究事業）
（総括担）研究報告書

新規磁性薬剤化合物の画像診断への応用に関する研究

主任　研究者　石川義弘　横浜市立大学大学院・教授

研究要旨

磁性物質は無機化合物というのが旧来の常識であった。ところが、エレクトロニクスの先端分野では「磁性を持った有機化合物」が多数研究・報告されている。これらはまだ医療に応用されていない。我々は石川島播磨重工業（株）基盤研究所との共同研究から、超伝導などの金属材料の研究に用いられる物理理論（第一原理解析方法）の応用により、「一般化合物に磁性を見つける方法」を開発した〔国際特許申請中〕。

我々がこれまでに磁性体と判定した化合物には市販の医療系薬剤が含まれており、直接の磁性測定によってもマグネタイトに匹敵する強磁性を有するものを見つけている。このことは、一般医薬品の中から磁性体を見つける可能性を意味する。さらに磁性医薬品は画像診断に利用できる。がん転移の画像診断や薬物の体内分布は放射性化合物を用いて行われてきたが、放射能の半減期や安全性、さらにサイクロトロンやPETをはじめとする設備の特殊性という問題があった。また医療用薬物、とりわけ抗がん剤の体内分布や代謝評価は，個人に合わせた効能や副作用の検討(オーダーメイド医療)において重要であるにもかかわらず，放射活性を用いて血中ないし尿・糞便中濃度を測定するといった原始的な方法に限定されてきた。そこで磁性体薬物を造影剤として用いれば、近年のMR画像技術の進歩と合わせて、がんの浸潤や転移の画像診断だけでなく、薬物の体内分布の検討も含めて可能となる。さらにこれらの磁性薬剤とMRIなど画像技術を結合させれば、従来困難とされていた生体の機能画像法を大きく発展させることができる。さらに、薬剤化合物の生物学的特性を、画像診断に結びつけることにより、従来困難とされてきた特定の臓器ないし組織機能診断のブレークスルーとなる。

平成１９年度においては磁性有機化合物の選定を行い、特定の磁性医薬品化合物が医薬効果を発揮することを培養細胞において証明し、さらに予定計画より早期に実験動物においてＭＲの造影剤としても機能しうることを検討した。この検討は、大学病院等において使用されている一般的なＭＲＩ装置を用いて、ファントム実験をおこなって実証し、さらに動物モデルにおいて小動物用のＭＲＩ撮影装置を用いておこなった。これらの結果から、有機磁性体をＭＲにおける造影剤として開発する可能性が裏付けられた。

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A．研究目的

一般に磁性体とは磁性を帯びる物質をさし〔いわゆる磁石〕、酸化鉄（マグネタイト）など無機化合物が代表的である。エレクトロニクス分野では、電動機の界磁として利用したり、磁性粉をテープ上に塗布して磁気記録に用いられる。

このような磁性体は、かつての常識では無機物質に限られ、有機化合物に磁性体が存在することはないとされてきたが、エレクトロニクスや材料分野では有機化合物でも磁性を帯びた物が開発・発見されており、現在では「磁性体は無機物に限定されない」というのが共通認識となっている。

有機磁性体を見つける方法を特許化

ところが、どうやって効率よく有機磁性体化合物を見つけるかが技術的問題であった。我々は物理理論（第一原理解析）の応用により、有機化合物が磁性体であるかどうかを簡便に判定する方法を開発した〔国内および国際特許申請中〕。

有機磁性体を医療に応用する

本申請では、世界的に研究が進み、わが国においても主導的な研究が行われている有機磁性体研究を、エレクトロニクスへの応用でなく、医療における画像診断へ応用する。独自の特許技術を用いるため、独占的な技術開発が可能となる。

期待される効果

有機化合物は無機化合物に比較して、圧倒的に生体適合性が高く、現在医療分野でニーズの高い安全な診断・検査薬品として有用である。さらに磁性を用いてがん組織に親和性の高い薬剤をもちいれば，がんの浸潤，転移など画像診断に応用することができ，さらに一般医薬品に磁性を有するものがあれば，既存のMRI施設等を用いて，一般病院においても薬物の体内分布や薬物動態を調べることが可能になる。前者は従来の放射性元素を使用したものに比して安全性・簡便性の高い画像診断として，後者は薬物動態の個人特性の評価に有用であり，薬物治療におけるオーダーメイド医療に重要な役割を果たすことが期待される．さらに生体内機能蛋白に親和性の高い有機化合物を用いれば，従来困難とされていた生体病変の機能を画像で診断する(バイオプシーなしで，がんの悪性度診断をするなど、いわゆる機能画像検査法)も可能になる。

本申請においては，我々自身が開発した特許技術を用いて，安全性の高い医療材料・薬品として，診断技術に応用可能な有機磁性体の開発を行うことを目的とする。我々のこれまでの研究成果において，すでに既存のがん治療薬に，マグネタイトに匹敵する磁性を有する有機磁性化合物を見出しており（特許申請中）、MR画像診断においても顕著な信号増強作用を確認し、造影剤として使用可能であることを見出している（別件特許申請中）。

B．研究方法

本申請の最終目標は，有機磁性化合物の画像技術応用であり，助成期間中の到達目標は，１）既存の医薬品の磁性評価を行い，２）画像診断への応用を非生体実験および動物実験として検討し、３）新規画像技術としての実用化を検証していくことである．平成１９年度はその第一年目として以下の研究方法を用いた。

１）医薬品の磁性評価

既存の医薬品化合物において，MR画像診断に有効性が期待される化合物を中心に，第一原理解析法を用いた磁性予測をおこなった．　対象化合物として、腫瘍の悪性度マーカーに結合する化合物を含む。

上記にて強度磁性が予測された化合物に関して，電子スピン共鳴装置を用いて実際の磁性強度の測定を様々な物理条件下で行う。世界標準機であるBruker Biospin社のEMXplusの装置の使用が不可欠であり、本申請の最大の出費申請項目として購入した。

２）MRによる非生体実験および動物実験

同定された候補化合物をもちいて、実際のMRにおける利用を、非生体および動物実験において検討する。非生体実験においてはMR器機を使用して、ファントムにおける温度、pH、溶媒条件などの指標をもちいて、画像信号の特異性を検討する。静磁場強度設定は国内における最強度の７テスラを当初目標として，最終的には一般病院における１．５テスラでのシグナル変化を検出することを目標とする．

非生体実験においてシグナル変化を確認した後、げっ歯類を用いた動物実験において画像診断候補化合物の造影効果の確認を中心にすすめる．とりわけ信号変化を正常動物において検討すること目的とする。

本申請では、製薬会社等で創薬事業に長年関与してきた申請者と、物理学者で材料研究をおこなってきた江口晴樹という、異分野の研究者が学際的な共同研究を組み、これにMR画像技術の開発を手がけてきた放射線科の李進とＮＩＨにて様々な薬理実験を手がけてきた黒谷玲子が同施設内の共同研究者として加わった。

C．研究結果

結果の概要

我々の開発した技術によれば、既存の化合物を含めて化学構造式の判明している化合物であるなら、すべてにわたって磁性の予測計算ができることがわかっている。いわゆる理論物理学の分野で使用されてきた第一原理計算法とよばれる手法であり、本手法によればすべての化合物を原子レベルにまで細かく分析し、各原子と電子の分布状態から電子スピン密度を計算し、得られた数値から磁性共同を予測する方法であり、超電導物質の開発等において繁用されてきた手法である。しかるにそのような計算は金属材料を主体とすることが多く、また同定された材料化合物に対しても磁性強度の測定は絶対零度に近い極低温でおこなわれるのが通常であった。これは分子運動に対する熱の影響を極力最低限とするためであり、また既存の超伝導はおなじく極低温下で初期検討を行うことが多かったためである。

１）医薬品の磁性評価

我々はこの課題に対して、第一原理解析による磁性予測を一般の化合物、とりわけ薬理学的作用を有すると考えられる物質にまで拡大して、磁性予測計算を施行した。その結果、従来は磁性体と考えられていなかった、一般の有機化合物の中にも、磁性予測上は強度の磁性を持つと考えられる化合物が複数存在することを見出した。続いて、これらの磁性が予測された化合物に対して、物理的な磁性測定をおこなっていったが、従来的な極低温での測定のみならず、３７度の体温相当環境下での測定をおこなった。これは本申請におけるＭＲ造影剤としての将来的な利用を考慮した場合、患者に投与した場合は３７度程度、実際の造影手技に関しては室温において磁性強度を有することがなければ、実用化は困難と考えたためである。実際の磁性強度測定においては、本測定の世界的標準手法となっているＳＱＵＩＤを使用した。実際に得られた磁場磁化曲線においては現有のマグネタイドに匹敵する磁性強度を有する化合物が同定された。さらに磁場磁化曲線における特性は、温度非依存性であり、室温においても極低温に相当する磁場磁化曲線が得られた。これらの事実から、有機磁性体化合物の磁性予測が実用的であり、実際にそのような化合物が存在し、さらにそのような化合物において室温において磁性の存在が確認されたことになった。

次にそのような有機磁性体化合物に対して、ＭＲ造影剤としての利用価値を検討した。旧来のＭＲ造影剤は無機金属物質が主体であり、金属の磁性体としての機能を造影剤として利用したに過ぎない。有機磁性体が存在するのなら、有機化合物としての機能を、ＭＲ造影機能に加味することができるはずである。

そこで我々はＥＩ２３６に対して、磁性以外の薬理作用があるかを検討した。我々は当初、本化合物の細胞増殖に対する作用について検討した。本化合物を細胞培養条件において、がん細胞に対する効果を検討すると、強い増殖抑制効果を有することが判明した。増殖抑制効果は用量依存性であり、高濃度においては細胞致死作用を有することがわかった。つまり、本薬剤は磁性をもった細胞増殖抑制剤であり、抗がん作用を有することがわかった。

我々は引き続き、この抗がん作用を磁場によって誘導できるかの検討を行った。培養フラスコの一角に市販の永久磁石を装置し、培養液中に抗がん剤を添加し、２４時間培養の後、永久磁石を付加した部位とその遠位部におけるがん細胞の増殖の程度を比較検討した。この結果、永久磁石近位部においては著名な細胞数の現象が見られたが、遠位部においては極めて盛んな細胞増殖が認められた。このことから、永久磁石による磁場によって、本抗がん剤が培養溶液中において移動し、濃度勾配を生ずることなり、永久磁石の近位部において高濃度に集積し、その抗がん作用を発揮することによって、がん細胞の増殖が抑えられたと推測された。このことは、有機磁性体のもつ抗がん作用を、磁場によって誘導することができることを意味した。

２）MRによる非生体実験および動物実験

我々はつづいて、有機磁性体によるＭＲ造影実験をファントムを用いておこなった。ＭＲ撮影装置としては附属病院において一般的に使用されている１．５テスラのＭＲ撮影装置を用いて、様々な濃度に調節した抗がん剤を、Ｔ１およびＴ２等に撮影条件を変えて、シグナル強度の変化を観察した。この結果、Ｔ１強調像において背景コントロール溶液に比して、極めて高いシグナルが得られることがわかった。このことはファントム実験において、本磁性有機化合物は造影剤として機能することがわかった。

我々は引き続き、ファントム中ではなく、生体内において本抗がん剤がＭＲ造影剤として機能するかの検討をおこなった。本抗がん剤の体内代謝速度および血中投与時の分布等は不明であったため、体内動態が比較的安定していると考えられる腹腔内への投与をおこない、直後において小動物用のＭＲ撮影装置を用いて様々な撮影条件下で検討をおこなった。えられた画像におけるシグナルを観察した結果、Ｔ１撮影条件下において鮮明に腹腔内臓器周辺が造影される事が判明した。このことは、本抗がん剤は成体においてＭＲ造影剤として機能することがわかった。

Ｄ．考察

本研究の目的は旧来は診断技術に使用されたことの無い、新規化合物をＭＲ撮影における造影剤として開発するものであり、その点において、生物的作用を持つ薬剤がＭＲ造影剤として機能しうることを実証した本年度の研究成果は価値が高いと考えている。

旧来はＭＲ造影剤はガドリニウムなどの金属体がすべてであり、造影剤自体には機能を持つものが無かった。これは金属体を造影剤として使用する以上、金属としての性質しか利用することができず、応用上は避けることのできない問題とされていた。もしも何らかの機能を持った医薬品化合物が、ＭＲにおける造影機能を有するとすれば、機能を有する造影剤と言う点において、将来的な医療応用、とりわけ画像診断における利用価値が極めて高いものになると考えられる。

本年度の研究成果は、この点において画期的な一歩を踏み出すことができたと考えられる。我々が同定した有機磁性体化合物のひとつに抗がん剤がある。生物学的な作用としては、細胞増殖抑制作用を有し、いわゆる抗がん剤としての薬理学的効果を示すことがわかっている。本年度の実験結果によれば、本薬剤はＭＲの造影剤として機能することが、小動物を用いたＭＲ撮影実験から判明した。このことは将来的なＭＲ薬剤開発の方向性に大きな影響と与える。抗がん剤の投与の決定は体表面積によるものが主体であり、具体的に患部（癌組織）にどの程度の抗がん剤が移行したのかを定量する方法は存在していなかった。もしも抗がん剤がＭＲ造影剤として機能すれば、ＭＲ撮影によって癌組織における抗がん剤の分布量が定量可能となり、今後の抗がん剤治療に画期的な進歩をもたらす可能性がある。

E．結論

有機磁性体化合物をＭＲ造影剤として、新規画像診断技術として開発していくことは可能であると考えられる。

F．健康危険情報

特記すべきこと無し。

G． 研究発表

1． 論文発表

Eguchi H, Iwatsubo K, and **Ishikawa Y**: Isoform-selective regulation of adenylyl cyclase by forskolin derivatives: Prediction of selectivity by computer-based analysis. ***Letters in Drug Design & Discovery*** 4:434-441, 2007

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2． 学会発表

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H．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

なし（検討中）

2．実用新案登録

なし

3．その他

別紙5

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Eguchi H, Iwatsubo K, **Ishikawa Y**	Isoform-selective regulation of adenylyl cyclase by forskolin derivatives: Prediction of selectivity by computer-based analysis.	***Letters in Drug Design & Discovery***	4	434-441	2007
Ishikawa Y, Suzuki S, Ohtsu K, Coskun Ulucan, Iwatsubo K, and **Eguchi H**	cAMP-mediated regulation of CYP enzyme and its application in chemotherapy.	***Drug and Metabolism Letter***.	1	176-178	2007

Letters in Drug Design & Discovery, 2007, 4, 434-441

Isoform-Selective Regulation of Adenylyl Cyclase by Forskolin Derivatives: Prediction of Selectivity by Computer-Based Analysis

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Abstract: Adenylyl cyclase is a membrane-bound enzyme that catalyzes the conversion of ATP to cAMP upon various hormonal stimulations. Isoform-selectivity among forskolin derivatives that forskolin and its derivatives are a direct activator of adenylyl cyclase, can be predicted mostly by the distribution of the negative electrostatic potential of each derivative.

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Isoform-Selective Regulation of Adenylyl Cyclase by Forskolin Derivatives: Prediction of Selectivity by Computer-Based Analysis

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Abstract: Adenylyl cyclase is a membrane-bound enzyme that catalyzes the conversion of ATP to cAMP upon various hormonal stimulations. Isoform-selectivity among forskolin derivatives that forskolin and its derivatives are a direct activator of adenylyl cyclase, can be predicted mostly by the distribution of the negative electrostatic potential of each derivative.

Keywords: Adenylyl cyclase, Isoform-selectivity, cAMP, Forskolin, Electrostatic potential, First principles calculations.

INTRODUCTION

Adenylyl cyclase (AC) is a membrane-bound enzyme that catalyzes the conversion of ATP to cAMP upon stimulation of numerous hormonal receptors, and thus plays an important role in regulating function of body organs [1]. It is known that there are at least nine isoforms (types 1-9) of AC that differ in tissue distribution and biochemical properties. Forskolin is a natural plant that has been used in traditional medicine in India [2]. Forskolin directly activates AC and thus because of this it acts like a β-adrenergic agonists, widely used drugs for acute heart failure. Forskolin increases ventricular contractility and induces vasodilatation in animals *in vivo*, and thus it was once considered for the treatment of acute heart failure as well. Despite such expectations, however, forskolin was never used as an alternative to β-adrenergic agonists in modern medicine because it has multiple side effects, most of which result from poor AC isoform-, and thus organ-selectivity [2].

More recently, a new forskolin derivative, or NKH477, has been developed , which lacks the side effects and is widely used in the treatment of acute heart failure in Japan [3]. This compound is known to possess high selectivity to the type 5 isoform [4], a dominant AC isoform in adult hearts, and thus selectively activates cardiac AC, leading to enhanced cardiac contractility. Accordingly, forskolin derivatives with increased AC isoform selectivity can be used in the treatment of diseases, in which catecholamine signal needs to be activated in an organ-dependent manner.

AC isoform selectivity of forskolin derivatives can be examined, at least in part, by utilizing information from crystallographic studies. Recent crystallographic studies have demonstrated the molecular mechanism of forskolin-mediated activation of AC; forskolin directly binds to AC at the opposite end of the site, to which ATP binds within the catalytic core [5]. Forskolin binds the two domains (C1 and C2) of AC by a combination of hydrophobic and hydrogen interactions, increasing the catalytic rate of this enzyme. Crystallographic studies also showed that there is a relatively large open space between the C6 and C7 residue positions of forskolin within its binding site of AC. In a previous study from our laboratory, we synthesized more than 200 new forskolin derivatives and have demonstrated that the modification at the positions of C6 and C7 indeed enhanced AC isoform selectivity [6]. For example, when the C6 position of forskolin was modified to an α-, β-unsaturated carbonyl group (6-(4-acrylbutyryl) forskolin), it selectively stimulated type 2 AC. The polar substitution at the C7 position as well as the attachment of C-C double bonds to the ring core of forskolin (the C5 and C6) increased type 3 AC selectivity. Mechanisms of interaction between such derivatives and AC isoforms were also confirmed by virtual docking computer analysis using results from crystallographic studies [6].

The above analysis was based on classical mechanics using empirical parameters that would reproduce *in vitro* pharmacological assays, which are very labor intensive and time consuming. In terms of accuracy of molecular interactions, particularly for the involvement of charge transfer and polarization, the classical molecular dynamics analysis commonly employed in the virtual docking study may not be always reliable. We were thus interested in applying a quantum mechanics based analysis, or first principles calculations, to the above process, which does not require intensive *in vitro* pharmacological assays. The first principles calculations have been used to determine the interaction between ligand and receptor by treating full atoms within the analyzing system. However, such a system has an extremely high degree of complexity because the system typically contains thousands of atoms of receptor proteins. In the current approach, however, we have analyzed forskolin derivatives themselves, and examined potential differences among six forskolin derivatives, which have been extensively examined in our laboratory for AC isoform selectivity [6]. We have employed both non-local and local analyses. Non-local analysis is not dependent on the position, such as binding energies, the first ionization

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energy, the electron affinity and the HOMO (Highest Occupied Molecular Orbital)-LUMO (Lowest Unoccupied Molecular Orbital) gaps, while local analysis includes dependency of the position, such as electrostatic potentials and distribution maps of the HOMO and the LUMO for analysis of the derivatives. We will demonstrate the mechanisms, by the use of quantum mechanics based analysis, that how the modification at the positions of C6 and C7 produces AC isoform selectivity, and the feasibility of applying this method to predicting AC isoform selectivity.

MATERIALS AND METHODS

1. Forskolin and its Derivatives

We have analyzed forskolin and its derivatives that have high AC isoform selectivity as shown in detail in a previous study [6] (Table 1). All derivatives are modified at R6, R7 and/or R13 residues. Briefly, FD1 is 6-[N-(2-isothiocyanatoethyl)aminocarbonyl] forskolin; the relative potency of stimulation of FD1 *versus* forskolin was 219 % for type 2, 46 % for type 3, and 21 % for type 5 AC. FD2, 6-(4-acrylbutyryl) forskolin (117 % for type 2, 59 % for type 3, and 23 % for type 5 AC); FD3, 7-deacetyl-7-hydroxamylforskolin (108 % for type 2, 221 % for type 3, and 94 % for type 5 AC); FD4, 5,6-dehydroxy-7-deacetyl-7-nicotinoylforskolin (116 % for type 2, 307 % for type 3, and 77 % for type 5 AC); FD5, 6-[3-(dimethylamino) propiony]forskolin (NKH477) (109 % for type 2, 72 % for type 3, and 180% for type 5 AC); FD6, 6-[3-(dimethylamino) propionyl]-14 15-dihydroforskolin (51 % for type 2, 22 % for type 3, and 139 % for type 5 AC).

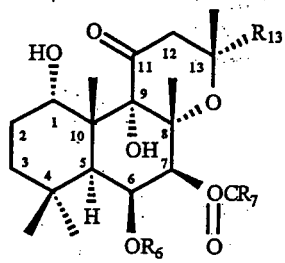
2. Methods of Calculation

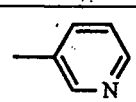
Calculations listed below have been performed using the PC cluster (Bestsystems, Tsukuba and Tokyo, Japan) as parallel computing of Advanced Applied Science Department in Ishikawajima-Harima Heavy Industries Co. Ltd. We have used software based upon Dmol^3 [7] (Accelrys, San Diego, CA, U.S.A.) by the use of Linux operating system of Redhat ES 4.0 (Redhat Inc., Raleigh, NC, U.S.A.).

3. Computational Details

We have calculated forskolin and its derivatives by linear combination of atomic orbital (LCAO) with spin polarized calculation. We have performed all electron calculation with double numerical basis-set including polarization function [7, 8] using the discrete variational method (DVM) [9-11]. A finite basis-set cutoff of 4.0 Å was used to reduce computational time without any significant loss in accuracy [12]. The local density approximation (LDA) [13] and the generalized-gradient approximation (GGA) by Perdew, Burke and Ernzerhof (PBE) [14] and Becke, Lee, Yang and Parr (BLYP) [15, 16] have been applied to obtain exchange-correlation energy functional. The GGA functionals depend

Table 1. The Chemical Structure of Forskolin and its Derivatives (6)



Compound	Position			Selectivity
	R_6	R_7	R_{13}	
Forskolin	H	CH_3	$CH{=}CH_2$	Non-selective
FD1	$CONHCH_2CH_2NCS$	CH_3	$CH{=}CH_2$	Type 2 AC
FD2	$COCH_2CH_2CH_2COCH{=}CH_2$	CH_3	$CH{=}CH_2$	Type 2 AC
FD3	H	NHOH	$CH{=}CH_2$	Type 3 AC
FD4	5,6-dehydoxy		$CH{=}CH_2$	Type 3 AC
FD5 (NKH477)	$COCH_2CH_2N(CH_3)_2$	CH_3	$CH{=}CH_2$	Type 5 AC
FD6	$COCH_2CH_2N(CH_3)_2$	CH_3	CH_2CH_3	Type 5 AC

The abbreviation of forskolin derivatives used in the text and the modification of their residues at R6, R7, and R13 are shown. FD1, 6-[N-(2-isothiocyanatoethyl) aminocarbonyl] forskolin; FD2, 6-(4-acrylbutyryl)forskolin; FD3, 7-deacetyl-7-hydroxamylforskolin; FD4, 5,6-dehydroxy-7-deacetyl-7-nicotinoylforskolin; FD5, 6-[3-(dimethylamino)propiony]forskolin (NKH477) ;FD6, 6-[3-(dimethylamino)propionyl]-14 15-dihydrofprskolin. The structure of forskolin and the position of each residue modified (R_6, R_7, and R_{13}) are indicated.

on both the local electron charge density and the magnitude of its gradient. The idea of the functionals is similar between PBE functional and BLYP functional although PBE functional is applied to the bulk system and BLYP functional to a molecule in general.

In order to evaluate the structures of forskolin and its derivatives, i.e. optimized structures, wavefunctions and eigenvalues, we utilized the following methods. A molecular structure was set according to the chemical formula of the forskolin and its derivatives, followed by dynamic optimization of the structure without empirical parameters. The structural and electrical optimizations were performed by the direct inversion in the iterative subspace (DIIS) [17, 18]. The derivatives were optimized by minimizing a total energy up to 2.7×10^{-4} eV and relaxed by minimizing a force acting on an atom up to 0.54 eV/nm. After determining the Kohn Sham equation that can approximate Schroedinger equation, we evaluated physical and chemical properties of these compounds through wavefunctions and eigenvalues.

The first ionization potential and the electron affinity of a molecule are calculated using density functional method [19]. The method can be expressed by total energy differences between two different states. There are,

$$IP = -(E_N - E_{N-1}) \quad (1)$$

and

$$EA = -(E_{N+1} - E_N) \quad (2)$$

where IP is the first ionization potential, EA the electron affinity, E_{N+1} the total energy of the molecule with the number of $N + 1$ electrons, E_N the total energy of the molecule with the number of N electrons and E_{N-1} the total energy of the molecule with the number of N-1 electrons. The binding energy is calculated as

$$E_b = E_{tot} - \sum_i E^i_{atom} \quad (3)$$

where E_b is the binding energy of the molecule, E_{tot} the total energy of the molecule and E^i_{atom} is the total energy of the i^{th} isolated atom [20-26].

RESULTS

We have analyzed forskolin and its derivatives (FD1 to FD6), of which AC isoform selectivity has been studied in detail [6]. These derivatives have been subdivided into three groups, FD1/2, FD3/4, and FD5/6, which selectivity stimulate type 2, type 3 and type 5 AC isoforms, respectively [6] (Table 1).

1. AC Stimulating Activity Analysis with Non-Local Study

We first employed non-local analysis, which does not depend upon the position within the molecule, but rather analyzes the dynamic properties of an entire molecule using multiple parameters. The analysis does not address properties with respect to the position, such as activities of a specific site in the molecule. We have determined LDA and the generalized-gradient approximation (GGA) with respect to the different approximation of exchange correlation functional. GGA was determined by both PBE functional and BLYP functional so that we could predict the binding energy of molecule precisely compared to that of LDA. It is known that BLYP functional gives a quite accurate description of hydrogen-bonded systems compared to PBE functional [27-30].

a. The First Ionization Energy, the Electron Affinity and Energy Gaps of the HOMO and the LUMO

Determination of the HOMO and the LUMO is based upon the frontier orbital theory, and is a method of calculating molecular orbitals [31-35]. According to Koopman's theorem, the energy level of the HOMO is directly related to the first ionization potential and the energy level of the LUMO, in contrast, is directly related to the electron affinity[36, 37]. The first ionization potential of the molecule is the energy required to remove one electron in a molecule. The electron affinity is the amount of energy absorbed when an electron is added to a neutral molecule to form an ion with a -1 charge ($-e$). It has a negative value if energy is released. When both the HOMO and the LUMO energies are related to the chemical interaction, those are called as radical interactions. For example, hard nucleophilies have a low energy HOMO; soft nucleophiles have a high energy HOMO; hard electrophiles have a high energy LUMO; and soft electrophilies have a low-energy LUMO. The HOMO-LUMO gap, i.e. the difference in energy between the HOMO and the LUMO, is an important stability index. The reason can be explained by the absolute hardness of a chemical species. The absolute hardness of a chemical potential $\mu[N, v]$ [38] is defined as,

$$\eta = \frac{1}{2}\left(\frac{\partial \mu}{\partial N}\right)_v = \frac{1}{2}\left(\frac{\partial^2 E}{\partial N^2}\right)_v \quad (4)$$

where η is the absolute hardness of a chemical species, E the total energy, N the number of electrons, v the external potential that is defined in density functional theory [19]. η measures the resistance to charge redistribution after the interactions. When hardness is small, it is easy for electrons to go to the others or come from the others. η is equivalent to the HOMO-LUMO gap through the finite difference approximation [19]. A large HOMO-LUMO gap implies high stability for the molecule because of its lower affinity in chemical interactions, and a small HOMO-LUMO gap implies low stability. The HOMO-LUMO gap has thus been used as an approximation to the excitation energy of the molecule.

We first thought that the isoform selectivity might be determined by the gap between the HOMO and the LUMO. We first calculated the first ionization energy and the electron affinity for each derivative using GGA (BLYP) and then the HOMO-LUMO gap (HOMO minus LUMO) by the methods of LDA and GGAs (BLYP and PBE).

Table 2 shows the first ionization potential and the electron affinity of forskolin and its derivatives with BLYP exchange correlation potential. Those energies were variable among derivatives. The first ionization potential varied from 6.379 (FD2) to 7.108 (FD4), and the electron affinity varied from 0.240 (forskolin) to 1.576 (FD2). When FD1 and FD2 were compared, the electron affinity of FD1 was lower than that of FD2, suggesting that FD1 can easily accepts electrons. The first ionization energy of FD1 was higher than

Table 2. The First Ionization Potential and the Electron Affinity (eV) of Forskolin and its Derivatives with BLYP Exchange-Correlation Functional

	Forskolin	FD1	FD2	FD3	FD4	FD5	FD6
Ionization potenitial (IP)	7.101	6.880	6.379	6.705	7.108	6.496	6.429
Electron affinity (EA)	0.240	0.910	1.576	1.526	1.057	0.336	0.585

that of FD2, suggesting that FD1 poorly gives up electrons. FD1 may interact with type 2 AC *via* acceptor like interactions, whereas FD2 may interact with type 2 AC *via* donor like interactions. Similarly, FD4 may interact with type 3 AC *via* acceptor like interactions, and FD3 may interact with type 3 AC *via* donor like interactions. When FD5 and FD6 were compared, the electron affinity of FD5 was slightly lower than that of FD6, and the first ionization energy of FD5 was slightly higher than that of FD6. Because both FD5 and FD6 contain a tertiary amine at R6, and the amine of FD5 and FD6 has a nucleophilic character due to a lone electron pair of nitrogen atom, FD5 and FD6 may have lower electron affinity.

We then compared the HOMO-LUMO gap of each derivative (Table 3). The gap was very different between FD2 and FD1, and the gap of FD2 was smallest among all the derivatives, suggesting that FD2 is the most reactive compound. The gap was modestly different between FD3 and FD4 while the gap was similar between FD1 and forskolin as well as between FD5 and FD6. The distribution map of the HOMO and LUMO in space was very similar between FD5 and FD6. Fig. (1) shows isosurfaces of the HOMO and the LUMO of FD5. The HOMO located at the R7 residue and the LUMO at the C9, the C10 and the C11 positions, and the modification at these residues is known to inactivate this derivative. Fig. (2) shows isosurfaces of FD6, which are very similar to those of FD5.

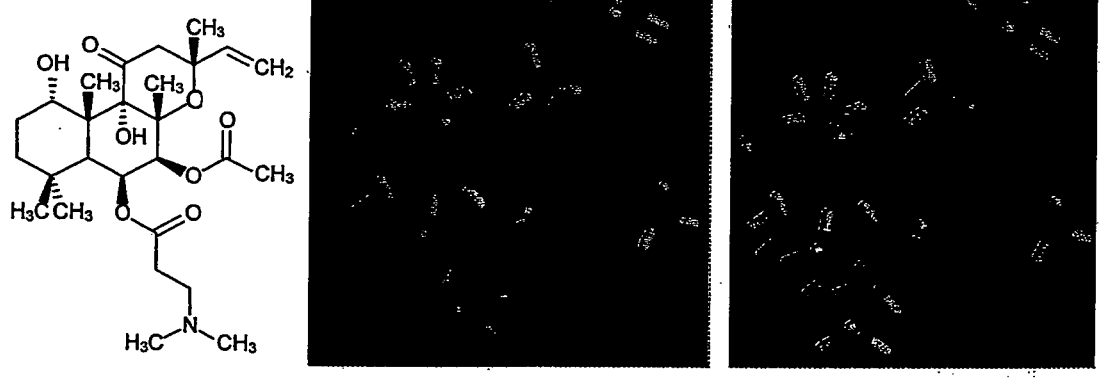
Putting together, the above findings suggest that AC isoform selectivity is less likely to be determined by the HOMO-LUMO gaps of these derivatives, at least determined by the first ionization energy and the electron affinity.

b. Binding Energies

We also analyzed the binding energy of each of forskolin derivatives because it was possible that the isoform selectivity among such derivatives might simply reflect differences in binding energy. We have also determined the value of the water molecule as a reference, which is known to have a very low affinity. As shown in Table 4, these derivatives had relatively small (below –4.425 eV/atom) and very similar (-5.160 eV/atom ~ -5.444 eV/atom by LDA and –4.425 eV/atom ~ -4.855 eV/atom by GGA) values in the

Table 3. The HOMO-LUMO Gaps (eV) of Forskolin and its Derivatives. In General, PBE Functional is Applied to the Bulk System and BLYP Functional to a Molecule

	Forskolin	FD1	FD2	FD3	FD4	FD5	FD6
LDA	2.914	2.962	1.521	3.129	2.855	3.194	2.910
GGA (BLYP)	3.147	3.224	1.628	3.412	3.043	2.801	2.908
GGA (PBE)	3.107	3.163	1.505	3.314	3.041	2.844	2.960



(a) HOMO (b) LUMO

Fig. (1). Isosurface of HOMO (left) and LUMO (right) of FD5. The positive value is shown in blue and negative in yellow. Chemical structure of FD5 is also shown (left).

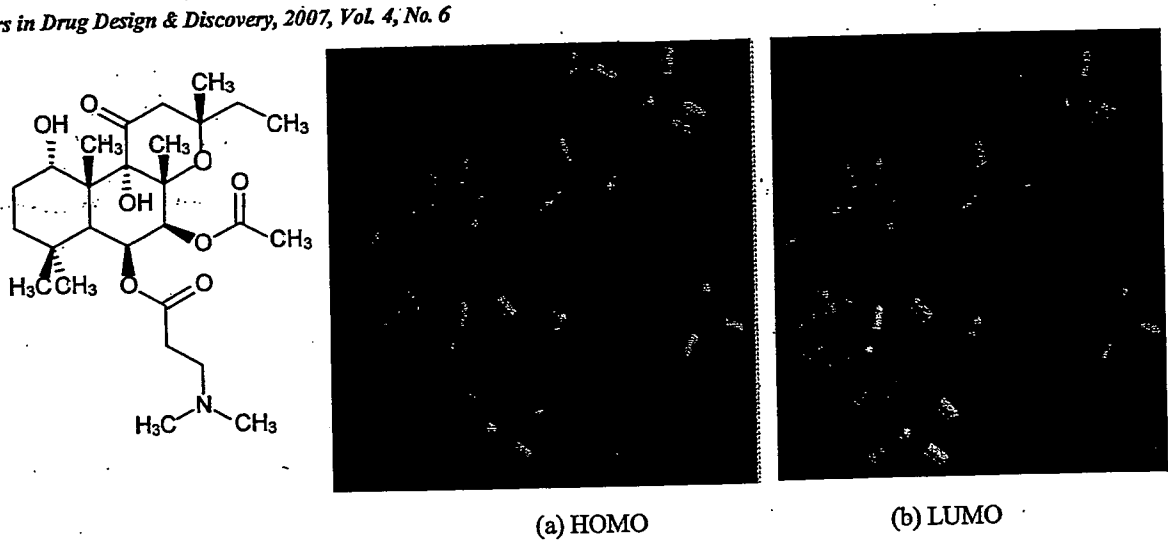


Fig. (2). Isosurface of HOMO (left) and LUMO (right) of FD6. The positive value is shown in blue and negative in yellow. Chemical structure of FD6 is also shown (left).

binding energy among the three groups (FD1/2, FD3/4, and FD5/6), indicating that the stability of these derivatives is similar at least in binding. Note that the value of the water molecule was relatively large (-3.817 eV/atom by LDA and -3.406 ~ -3.444 eV/atom by GGA) compared with those of the forskolin derivatives. These data suggest that the isoform selectivity is less likely due to the differences in binding energy.

Thus, the analysis using the HOMO and the LUMO has suggested the complexity of the mechanisms of generating AC isoform selectivity that molecular dynamics are not necessarily similar even between the derivatives with comparable isoform selectivity.

2. AC Stimulating Activity Analysis with Local Analysis

We next employed local analysis, which is suitable for looking at local positions of a molecule. The analysis addressed properties with respect to the position, such as activities of specific site in the molecule.

a. Isosurfaces of Electrostatic Potential

We have used the generalized-gradient approximation (GGA) throughout calculations on isosurfaces of electrostatic potential. Since all calculations on isosurfaces of electrostatic potential by the generalized gradient calculations (BLYP and PBE) were consistent with those of LDA, we used BLYP for calculating electrostatic potentials as described below. We calculated the electrostatic potential of each forskolin derivative because such potential might determine the affinity of each derivative to a specific AC isoform. The electrostatic interaction is represented by both the atomic charges and the positive point charges in space, such as within a grid surrounding the molecule, and can be either attractive or repulsive. For example, an electropositive part of a forskolin derivative seeks to dock to an electronegative part of an AC isoform.

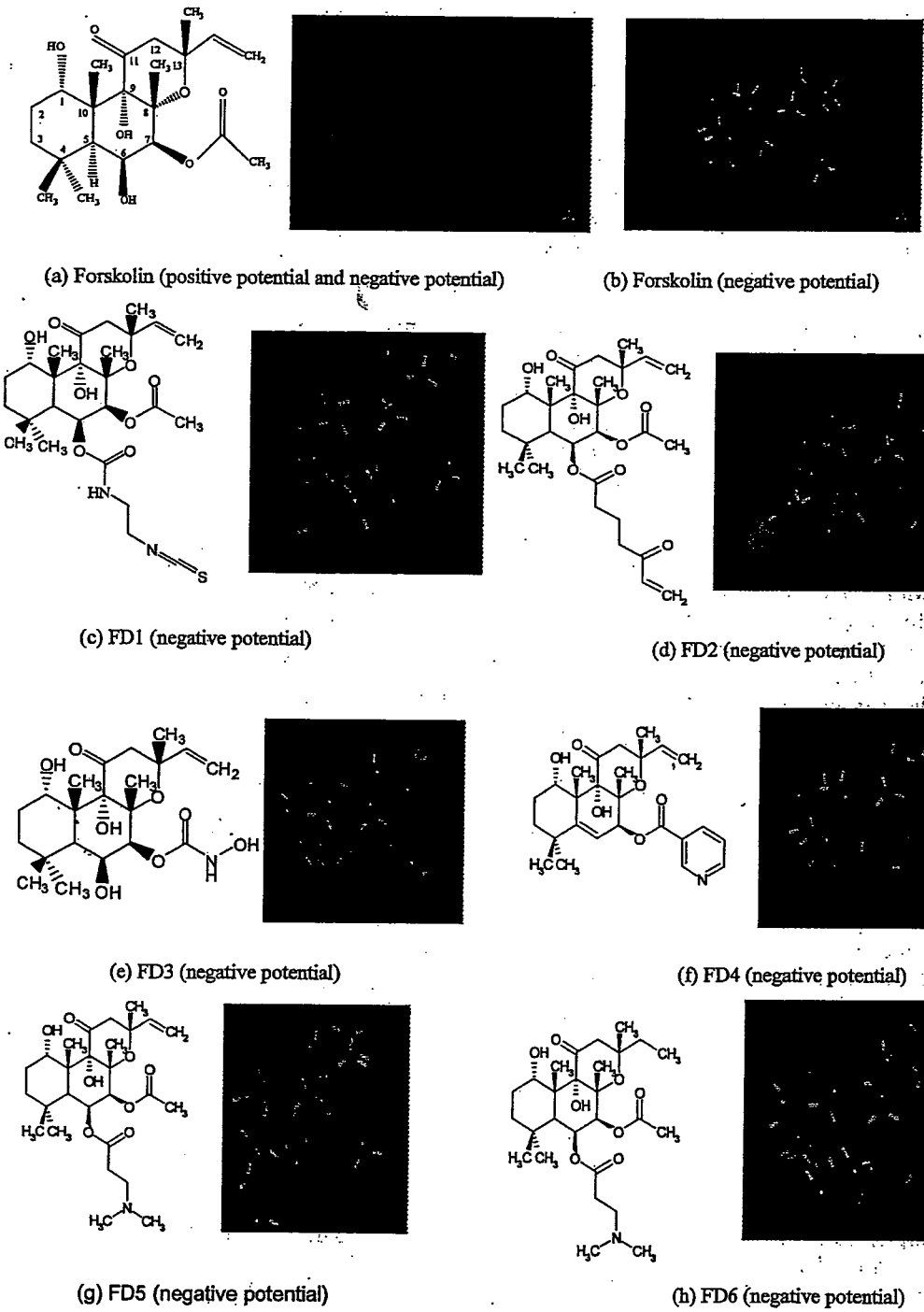
Isosurfaces of the electrostatic potential are summarized in Fig. (3). An isosurface is a three-dimensional analog of contour map. It can represent a surface of constant value of electrostatic potential within volume. Positive potential that takes higher value inside is shown in blue and the negative one that takes lower value in yellow. All isosurfaces are drawn at 5.880×10^{-4} eV in the positive and -5.880×10^{-4} eV in the negative.

Positive potentials were widely distributed over the molecule while the negative potentials were split into two regions in all derivatives. An example of forskolin is shown in Fig. (**3a**). The distribution of positive potentials of forskolin derivatives was mostly the same to that of forskolin, suggesting that such distribution is less likely to contribute to the isoform-selectivity. However, we found that the distribution of negative potentials was very different among them, but was conserved, importantly, between the derivatives with similar AC isoform selectivity as shown in Figs. (**3b-h**). FD1 and FD2, for example, which are selective to type 2 AC, had the negative potential spreading largely on the reverse side of the C6 and C7 positions but, to a smaller degree, on the front of the C7 position. The substituent of FD1 at R6 contains an isothiocyanate, which act as electrophilies with carbon atom as the electrophilic

Table 4. The Binding Energies (eV/atom) of Forskolin, its Derivatives and Water. In General, PBE Functional is Applied to the Bulk System and BLYP Functional to a Molecule

	Forskolin	FD1	FD2	FD3	FD4	FD5	FD6	H_2O
LDA	-5.203	-5.265	-5.290	-5.191	-5.444	-5.201	-5.160	-3.817
GGA (BLYP)	-4.497	-4.518	-4.551	-4.425	-4.643	-4.472	-4.437	-3.406
GGA (PBE)	-4.662	-4.689	-4.718	-4.600	-4.855	-4.634	-4.597	-3.444

center. On the other hand, the substituent of FD2 at R6 contains an α/β unsaturated ketone, which has a polar character. The results show FD2 of the R6 substituent $COCH{=}CH_2$ out of $COCH_2CH_2COCH{=}CH_2$ had the same character of the substituent of FD1 at the R6. This implicated that the positive electrostatic potential of type 2 AC [27] interacts FD1 and FD2 mostly at the reverse side of the C6, C7 and R7 positions. FD3 and FD4 are selective to type 3 AC and its negative potentials were entirely wrapping the C7 position, implicating that the positive electrostatic potential of type 3 AC interacts with FD4 at the C7 position from any directions. FD5 and FD6 is selective to type 5 AC and its negative potentials spread widely on the reverse side of the C8, the C7 position while they were weak at the C6 position, implicating that the positive electrostatic potential of type 5 AC interacts mostly with the reverse side of the C8, the C7 positions, and the C7 position.



(a) Forskolin (positive potential and negative potential)

(b) Forskolin (negative potential)

(c) FD1 (negative potential)

(d) FD2 (negative potential)

(e) FD3 (negative potential)

(f) FD4 (negative potential)

(g) FD5 (negative potential)

(h) FD6 (negative potential)

Fig. (3). Isosurface of electrostatic potential of forskolin and its derivatives. The positive value is shown in blue and negative in yellow, and chemical structure of each derivative is also shown (left). (a) Forskolin (positive potential and negative potential), (b) Forskolin (negative potential), (c) FD1 (negative potential), (d) FD2 (negative potential), (e) FD3 (negative potential), (f) FD4 negative potential), (g) FD5 (negative potential), (h) FD6 (negative potential).

The above findings suggest that the negative electrostatic potentials of these forskolin derivatives may interact with the positive electrostatic potential of each AC isoform, and that this interaction may play a potentially important role in producing isoform-selectivity among forskolin derivatives.

DISCUSSION

The isoform-selective stimulation of forskolin can be potentiated through specific modifications at the C6 and/or the C7 position of forskolin and that the combination of multiple modifications has additive effects in enhancing selectivity [6]. It is speculated that forskolin is a partial activator of AC and that the modification of forskolin at the C6 and the C7 positions may simply increase the maximal degree of AC activation. The affinity of our forskolin derivatives (FD1-6) for AC does not depend upon the structure at the C6 and C7 positions [6]. The hydrogen bonding with AC at the C1, the C7 and the C11 positions played an important role in the previous report [6]. Our analyses have revealed that the distribution of the negative electrostatic potential on the C6 and the C7 residues may play a role in type-specific regulation of AC by forskolin derivatives.

Analysis of forskolin derivatives using first principles calculations, which is based on quantum mechanic analysis, has revealed potential mechanisms for enhanced selectivity among forskolin derivatives. Most important, AC isoform-selectivity was best characterized by the distribution of the negative, but not positive, electrostatic potential of these derivatives, suggesting that the positive electrostatic potential of AC isoforms may play an important role in defining the interaction with these forskolin derivatives. In support of this concept, the negative electrostatic potential distributing over the C6 and C7 positions, of which modification is known to increase selectivity; was similar between the derivatives with comparable AC isoform selectivity, but very different among derivatives with distinct selectivity. Furthermore, presence of multiple mechanisms to generate AC isoform selectivity was suggested by the analysis using the first ionization potential and the electron affinity because these indexes were different even between the derivatives with comparable isoform selectivity. Acceptor like interactions plays an important role in AC isoform selectivity among types 2, 3 and 5 since FD1 which selectivity stimulates type 2 AC, FD4 which selectivity stimulates type 3 AC and together with FD5 which selectivity stimulate type 5 AC tend to have the lower electron affinity than the other counterpart.

Our study also demonstrated another potential advantage of employing the first principles calculations to analyze selectivity to enzyme isoforms. The first principles molecular dynamics has been used to determine the interaction, for example, between agonist and receptor by treating full atoms within the analyzing system. In the current study, we have focused our efforts on analyzing only agonists (forskolin derivatives) to examine if such analysis is sufficient to find properties that may correlate with AC isoform selectivity. The method of approach employed in our study was relatively simple because we did not have to analyze the AC protein that possesses a large number of atoms or utilized data from crystallographic studies. We do not know if the same approach can be applicable to other types of compounds that react with different effecter enzymes. However, our approach was indeed useful to predict AC isoform-selectivity, at least in part, among forskolin derivatives, and this may be used to design new forskolin derivatives at the time of molecule designing in future studies. Calculated properties like first ionization potential, electron affinity and HOMO-LUMO gaps could be useful indices if the mechanism of action of the forskolin and its derivatives could be related to a covalent or an ionic binding process. Our analysis also has a potential of further developing isoform-targeted, such as not only stimulators but also inhibitors without an extremely high degree of complexity calculations, such as full treatment of ligand and receptor. By the use of a similar approach, it may be possible to analyze the interaction between this enzyme isoform and other inhibitors, such as P-site inhibitors [39].

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ABBREVIATIONS

AC = adenylyl cyclase

FD1 = 6-[N-(2-isothiocyanatoethyl)aminocarbonyl] forskolin

FD2 = 6-(4-acrylbutyryl) forskolin

FD3 = 7-deacetyl-7-hydroxamylforskolin

FD4 = 5,6-dehydroxy-7-deacetyl-7-nicotinoylforskolin

FD5 = 6-[3-(dimethylamino)propiony]forskolin or NKH477

FD6 = 6-[3-(dimethylamino)propionyl]-14 15-dihydro-forskolin

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Preparation and Biological Activities of Heteroarotinoids

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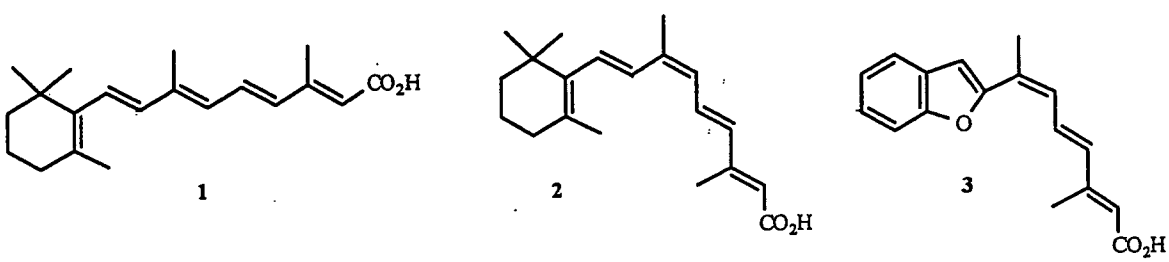
Abstract: Retinoic acid analogs containing an aromatic ring fixed between the 5 and 8 positions of retinoic acid, were synthesized by a palladium-catalyzed cross-coupling reaction between boronic acid and an alkenyltriflate or alkenyliodide. The biological activities of the retinoic acid analogs were evaluated.

Keywords: Heteroarotinoid, Boronic acid, Coupling reaction, RAR, RXR.

INTRODUCTION

Retinoic acid receptors (RARα, β, and γ) and retinoid X receptors (RXRα, β, and γ) are members of the nuclear receptor superfamily [1]. These receptors are ligand-dependent transcription factors that play important roles in a myriad of biological functions including cell differentiation, cell proliferation, and embryonic development [1,2]. Retinoids are natural or synthetic analogs of retinoic acid that act as signaling molecules by binding to RARs and RXRs. The natural ligand all-*E*-retinoic acid (ATRA, **1**) binds with high affinity to RARs but does not bind to RXRs. However, 9*Z*-retinoic acid (9CRA, **2**) exhibits nealy the same binding affinity for RARs and RXRs [3,4]. RXRs play important roles in heterodimeric complexes with other nuclear receptor proteins such as RARs, the thyroid hormone receptor (TR), the vitamin D receptor (VDR), and peroxisome proliferator-activated receptors (PPARs). Great efforts have been made to prepare a receptor-selective retinoids in order to define the functions of each receptor and to develop therapeutic agents [5]. We have demonstrated that the retinoic acid analog **3** (Scheme **1**), in which the cyclohexene and the adjacent C6-7 double bond of 9*Z*-retinoic acid is replaced with a benzofuran ring, causes increased binding of RXR to the retinoid X responsive element (RXRE), despite having no biological activities (antiproliferative, differentiation-inducing, or apoptosis-inducing) in HL-60 cells [6]. Recently, Michellys *et al.* reported that the analogs having benzofuran or benzothiophen ring exhibited good RXR selectivity [5i]. These findings prompted us to investigate the structure-activity relationship of retinoic acid analogs having a hetero-



Scheme 1. Structures of retinoic acid and analog.

aromatic ring (heteroarotinoids). This manuscript describes the preparation and the receptor-binding activities of new heteroarotinoids having furan, benzothiophene, and thiophene rings.

RESULTS AND DISCUSSION

Chemistry

Heteroarotinoids **9** and **9'** were synthesized using two types of highly stereroselective reactions for carbon-carbon bond formation (Scheme **2**). The first reaction is a Suzuki cross-coupling reaction of alkenyltriflate or alkenyliodide with boronic acid. This reaction is well known to proceed with retention of the double bond in the coupling components [7]. The reaction of boronic acids (**4**) with *E*-alkenyltriflate (**5**) or Z-alkenyliodide (**5'**) in the presence of tris(dibenzylideneacetone)dipalladium [$Pd_2(dba)_3$], triphenylarsine ($AsPh_3$), and sodium hydroxide afforded the coupling

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cAMP-Mediated Regulation of CYP Enzymes and its Application in Chemotherapy

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