Photosensitised Switchable Nitric Oxide Donor Furoxans

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論 文 内 容 の 要 旨

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専　　攻　　　Chemistry

論文題目（外国語の場合は，その和訳を併記すること。）

Photosensitised Switchable Nitric Oxide Donor Furoxans

光増感作用を利用した光応答性一酸化窒素ドナーフロキサンの開発

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## Photosensitised Switchable Nitric Oxide Donor Furoxans

Research summary

Nitric oxide (NO) is a reactive free radical with a short lifetime and area of effect. NO behaves as a cellular signalling molecule involved in vasodilation, cell death and neurotransmission. Diseases resulting from a deficiency of NO can be treated with stable NO donor molecules, however current clinical NO donors are non-specific and have issues with toxicity and tolerance.

Our research was focussed on the NO donor furoxan, which releases NO in presence of thiols. Previously it had been shown that fluorofuroxans can isomerize under UV irradiation (Scheme 1'). The 4-F regioisomer **1'** was stable to thiol but once isomerized to the 3-F regioisomer **2'** it underwent thiol mediated NO release. This previous research was the first example of furoxan photoswitchable NO donors (PINODs) but was limited by the fluorofuroxan absorption being within the UV range, which is damaging to organic tissue.
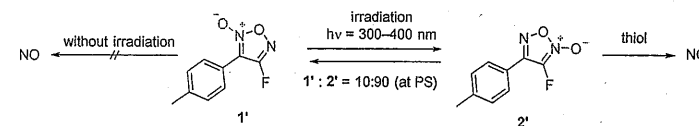
**Scheme 1':** Photoswitchable NO release from fluorofuroxan.

This set the precedent for the core of our research. We hypothesized that a visible light absorbing moiety may be able to transfer energy to furoxan and induce isomerization enabling NO release (Scheme 2'). During our initial investigations we discovered that furoxans could phosphoresce under irradiation, this suggested that furoxan could access the triplet excited state. The furoxan phosphorescence discovery highlighted photosensitizers as possible visible light absorbing moieties that could be conjoined with furoxan to form PINODs.
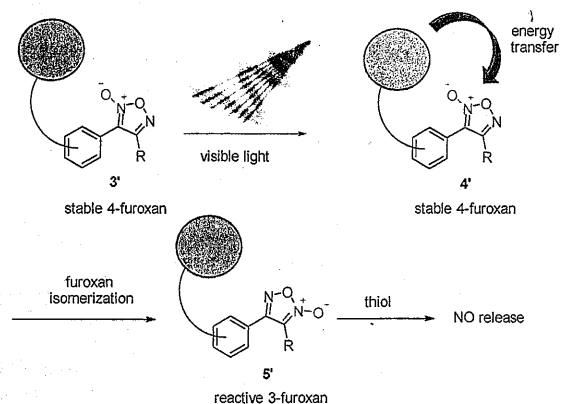
Scheme 2': Envisioned mechanism of furoxan isomerization and NO release mediated by visible light absorbing moieties.

Our first efforts into this research was in the search for a visible light absorbing photosensitizer of fluorofuroxan. Screening reactions of selected photosensitizers were conducted by irradiating a deaerated solution of deuterated benzene containing equimolar amounts of photosensitizer and fluorofuroxan with 400–500 nm of light. When photosensitization of the fluorofuroxan occurred, it underwent isomerization from **1'** to **2'** reaching a maximum isomerization ratio at the photostationary state (PS). We were able to observe this by comparing to the ^{1}H NMR spectra of irradiated and non-irradiated samples. We selected anthraquinone **6'** as the photosensitizer which induced up to 75% isomerization in the fluorofuroxan within 30 minutes of continuous irradiation (Scheme 3').
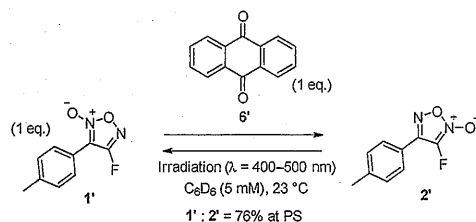
Scheme 3': Photosensitized isomerization of fluorofuroxan induced by visible light absorbing anthraquinone. Measured by ^{1}H NMR using relative peak integration.

With the anthraquinone sensitizer and fluorofuroxan partners selected, we devised synthetic strategies to unite them into a single hybrid molecule. Our first strategy utilized a direct nucleophilic substitution with ethyleneglycol functionalized anthraquinone **7'** onto a benzylic bromo-functionalized fluorofuroxan **8'**. Once the hybrid had been formed we intended to improve solubility

with longer glycol chains. Unfortunately, we observed incorrect regioselectivity with reaction occurring directly on the furoxan ring, conjoining the molecules but removing the essential fluoro-functionalization from the furoxan (Scheme 4').
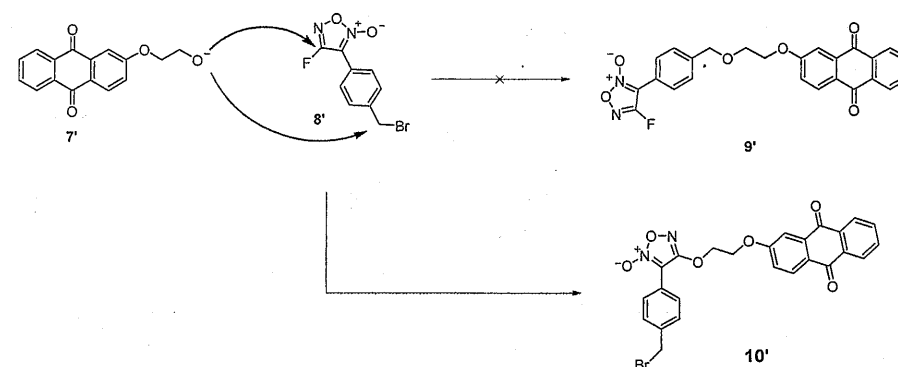

Scheme 4': Formation of undesired byproduct **10'** from reaction between ethyleneglycol functionalized anthraquinone **7'** and fluorofuroxan **8'**.

Our second attempt at connection made use of palladium catalysed cross coupling (Scheme 5'). Palladium coupling was selected in order to remove regioselectivity issues from the synthesis. Firstly, the Heck reaction between vinylanthraquinone **11'** and a *p*-bromophenyl substituted fluorofuroxan **12'** was attempted. However, we encountered stability issues with the fluorofuroxan which was predisposed to decomposition under the reaction conditions. We suspected that base was responsible for the instability of the furoxan so we altered our approach to the Stille coupling, which uses mild conditions in the absence of base. To our delight we were able to connect a *p*-stannylphenyl furoxan **15'** with a model β-iodovinylstyrene compound (not shown). We undertook extensive reaction optimization and significantly improved the yield. Finally, we synthesised an β-iodo-vinylanthraquinone **14'** coupling partner and applied our optimized conditions. We were able to connect the two coupling partners but the yield was poor and purification of the product **15'** was difficult. Further attempts at reaction optimization using the anthraquinone coupling partner were unfruitful. The difficulty encountered in isolating the product necessitated an alternative approach, one in which the anthraquinone core could be constructed late in the synthesis.
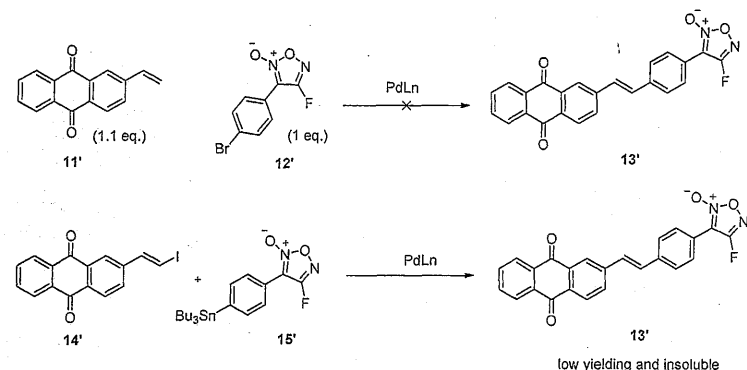
Scheme 5': Palladium catalysed coupling reactions between furoxans and anthraquinones.

Now that we understood the limitations of synthesising a furoxan-anthraquinone hybrid, we were able to mitigate the encountered problems of furoxan stability and anthraquinone insolubility. We planned to synthesise the anthraquinone core at a late stage in the synthesis whilst simultaneously connecting the furoxan in a single step. The envisioned reaction was an organocatalyzed [4 + 2] reaction between (relatively soluble) naphthoquinone and a terminal aldehyde functionalised furoxan **16'**. The initial attempts were troublesome due to stability issues with an azide functionalized naphthoquinone (not shown) so we changed the intermediate to an *N*-Boc protected analogue **17'** which assisted with the solubility and purification. Furthermore, the Boc group could be deprotected later in the synthesis with TFA to grant a water-soluble salt. The organocatalyzed [4 + 2] reaction furnished the anthraquinone core as well as the furoxan linker granting the desired product **18'** in moderate yield after purification (Scheme 6'). Deprotection and saponification of the *N*-Boc amine afforded the desired water-soluble hybrid anthraquinone-fluorofuroxan allowing us to test the photoswitchable nitric oxide donating ability.
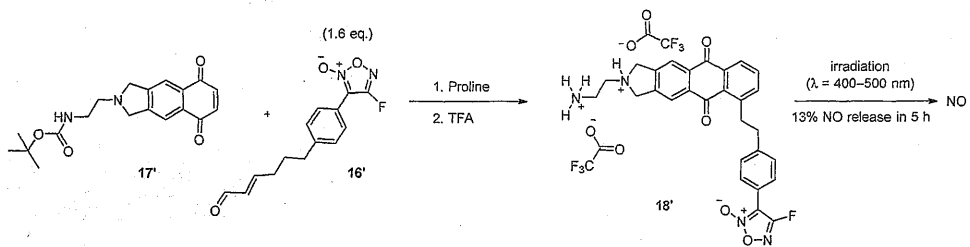

Scheme 6': Organocatalysed [4 + 2] reaction between naphthoquinone **17'** and furoxan **16'** followed by Boc deprotection and saponification.

Irradiation of the anthraquinone-furoxan hybrid **18'** in cysteine phosphate buffer resulted in photoswitchable nitric oxide release in up to 13% after 5 hours. Delighted that photoswitchable release had been achieved we set about improving both the rate and quantity of NO release.

To begin our investigation, we synthesised a disconnected anthraquinone molecule **19'** to test the

effect of the alkyl linker on the sensitization of fluorofuroxan. We irradiated a solution of deuterated DMSO containing equimolar concentrations of disconnected anthraquinone **19'** and fluorofuroxan **1'** with 400–500 nm light (Scheme 7'). To our surprise aside from inducing sensitization in the furoxan, the anthraquinone appeared to undergo oxidation to **20'** under irradiation and became fluorescent. This meant that once oxidized the photophysical properties of the anthraquinone changed and it may not be able sensitize the NO release from furoxan.
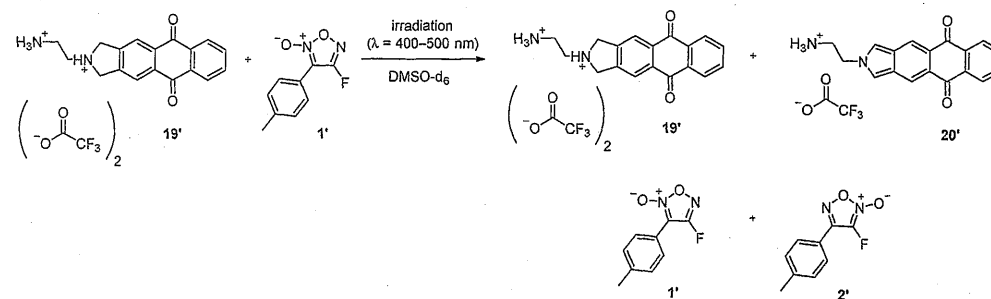
Scheme 7': Isomerisation of **1'** and oxidation of **19'** under visible light irradiation. Measured by ¹H NMR using relative peak integration.

To counter this phenomenon, we alkylated the pyrrole amine of **18'** with iodomethane and deprotected the Boc group to form **21'**, once the quaternary ammonium ion had formed, photooxidation would no longer be possible (Scheme 8'). Gratifyingly, when **21'** was irradiated with 400–500 nm light in cysteine phosphate buffer the nitric oxide release rate and quantity increased significantly to 39% after 5 hours. Furthermore, an unusual property of sensitized furoxan NO release was observed where NO was released under irradiation in the absence of cysteine. Very few furoxans can release NO in the absence of thiol cofactor, this is a limiting factor in furoxan NO donor design since the cellular levels of thiol vary. Cellular studies with **21'** were then conducted in the hopes that we could attain photoswitchable nitric oxide release from within a cell culture system. Unfortunately, we did not observe NO release from within the cells and therefore could not determine whether the hybrid had permeated the cell membrane.
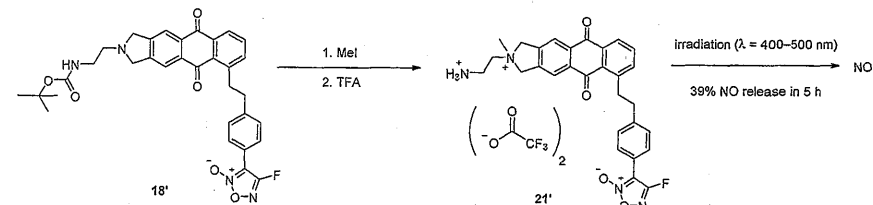
Scheme 8': Formation of furoxan-anthraquinone PINOD **21'** and NO release under irradiation.

The lack of cellular uptake was disappointing so the project progressed with a view to designing improved molecules to address the shortcomings of our initial prototype.

After considering the shortcomings of **21'**, we decided that an improved design would need to be accessible by a facile synthetic route and be observable by fluorescence (Figure 1'). This meant that the molecule must be theranostic (be able to fluoresce and sensitize) and that an alternative furoxan to fluorofuroxan was necessary.
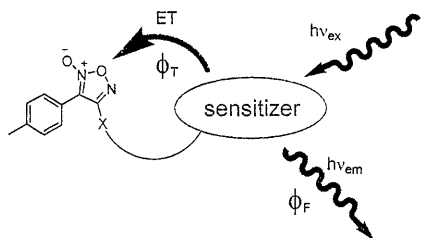

Figure 1': Concept for an improved sensitized furoxan PINOD.

We began our investigation by screening furoxans for their NO releasing ability under UV irradiation, under the assumption that visible light sensitization would produce similar quantities of NO. We found that alkoxyfuroxan was a good NO donor and a more stable alternative to fluorofuroxan. Our search efforts into fluorescent sensitizers led to 3-amino-9-fluorenone as a theranostic candidate. We synthesised a fluorescent fluorenone-alkoxyfuroxan hybrid **22'** which was successful at sensitizing isomerization of the furoxan in benzene. However, when irradiated in phosphate buffer solution there was no release of NO (Scheme 9').
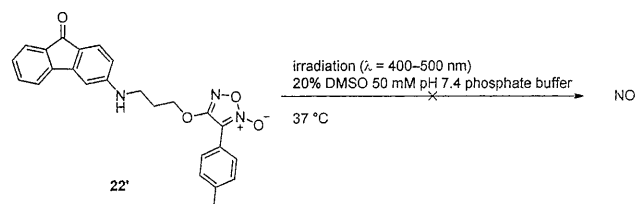

Scheme 9': Unsuccessful NO release from fluorenone-furoxan under visible light irradiation.

It appeared that the sensitized NO release of **22'** was quenched in aqueous solution. This was perhaps by rearrangement of hydrogen bonds around the fluorenone excited state or by destabilisation of the fluorenone triplet energy level making intersystem crossing difficult. We were pleased that our initial investigations were successful in organic solvent and searched for a system that would perform its function in aqueous solution.

Our search for visible light absorbing theranostic compounds led us the naphthalenediimides (NDIs). Recently NDIs had demonstrated singlet oxygen sensitization from within cancer cells leading to a

reduction in cell viability by up to 40% under irradiation. Encouraged by the water-solubility, fluorescence, cell permeability and sensitizing properties we designed an NDI tethered alkoxyfuroxan **23'** (Scheme 10'). To our delight the NDI-furoxan **23'** displayed photoswitchable NO release in phosphate buffer solution. However, when in the presence of cysteine, the NO release was prevented. This was possibly due to single electron transfer from thiol quenching the NDI excited state.
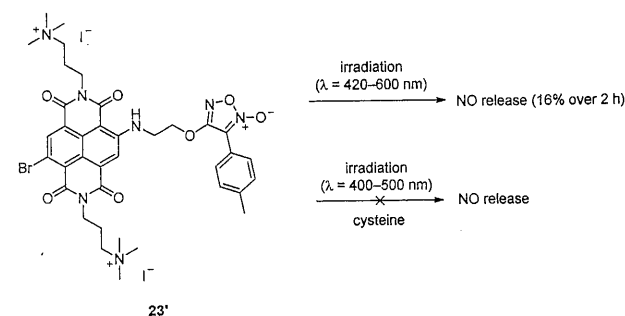
Scheme 10': In vitro investigation into NO release of **23'**.

Regardless of the lack of NO release in the presence of cysteine we tested the cellular uptake of **23'** in HeLa cancer cells (Figure 2'). We were clearly able to observe the fluorescence from within the cell showing successful cellular uptake and as expected, NO release was not observed due to the presence of quenching biothiols. We suggested synthetic modifications to the NDI-alkoxyfuroxan for future research to enhance the sensitized NO release of furoxans, hopefully in the presence of thiol.
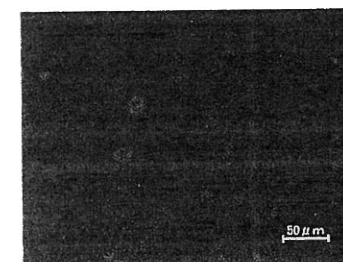

Figure 2': Cellular uptake of fluorescent compound **23'** in HeLa cancer cells.

In summary, we were able to synthesise successful in vitro furoxan-sensitizer PINODs but the designs were hindered by lack of biological application. We overcame the initial problems of cell permeability of anthraquinone-furoxan **21'** by designing an NDI-furoxan **23'**. We were able to observe cellular uptake of **23'** due to theranostic properties but NO release did not occur under irradiation.

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論文題目	Photosensitised Switchable Nitric Oxide Donor Furoxans 光増感作用を利用した光応答性一酸化窒素ドナーフロキサンの開発		
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<div align="center">要　旨</div>

　一酸化窒素は生体内において生合成され、シグナル伝達物質として重要な生理作用を担っている。その生理作用には、血管収縮作用、血小板凝集抑制作用、神経細胞におけるシナプス増強作用などが知られている。一酸化窒素の生理作用をターゲットとして、狭心症薬のニトログリセリンや勃起不全薬のシルデナフィルが既に開発されており、一酸化窒素の生理作用は今後ますます創薬のターゲットとして注目されると考えられる。しかしながら、一酸化窒素の適所への投与、すなわちターゲティングをする技術は不十分ではなく、さらなる開発が望まれている。

　Christopher Peter Seymour 氏の学位論文は、一酸化窒素の精密ターゲティングを目的として光に応答して一酸化窒素を放出する有機化合物の創製に取り組んだものである。光応答性一酸化窒素ドナーは、一酸化窒素を対象とした生理学実験の実験ツールとして有用であるだけでなく、将来的には光線力学療法への応用も期待される。このような背景の中、本論文はフロキサンというヘテロ芳香環に着目し、この化合物が光増感作用により光感受性一酸化窒素ドナーとして働くことを示し、さらに有機合成化学的な修飾を施し可視光領域にまで励起波長を長波長化させた成果をまとめたものである。

　本学位論文は、全体として七章から構成され、各章の内容は以下のとおりである。

　第一章では、一酸化窒素の生理作用について、作用機序を説明しながら述べている。さらに本論文で主に扱うフロキサン化合物の、歴史的背景、合成法、生理作用、一酸化窒素放出能などについて本論文で必要となる基礎的背景を詳述している。これらの知見を基にして、本論文における作業仮説と研究目的を説明している。

　第二章では、実際にフロキサン化合物の光増感作用について述べている。まず、フルオロフロキサンの燐光を測定した。弱い発光であったが 420 nm 付近に極大を持つ波形が得られ、フロキサンの三重項状態のエネルギー準位に関する情報を得た。その知見を基に種々の三重項増感剤をスクリーニングし、アントラキノンがフロキサンを効果的に励起できることを明らかにした。フロキサンを三重項増感機構により光励起した例はこれまでになく重要な発見と言える。次に、ここまでは増感剤とフロキサンは別々の分子として反応させていたが、双方を同一分子内に組み込むことにより光増感作用の効率化を図った。フロキサンの分子修飾は、フロキサン環の種々の反応条件における不安定性のために制限が多い。その制限の中、パラジウムを触媒としたカップリング反応条件がフロキサンに適用可能であることを示した。有機分子触媒を用いる環化反応を鍵反応としフロキサン−アントラキノン一体型分子を合成した。この分子は紫外光または可視光に応答して一酸化窒素を放出することを明らかにし、計画した通りの光物性を有していたと述べている。しかしながら細胞内での一酸化放出を目的とし細胞に投与したところ、細胞内で一酸化窒素を放出している明確な証拠を得ることができなかった。

　第三章では、第二章での問題点を改善する検討について述べている。合成した分子から一酸化窒素放出が観測されなかったのは分子が細胞膜を透過できなかったことが原因であった可能性を考えた。そこで光に応答して一酸化窒素を放出する性質の他に、蛍光性で分子の細胞内局在を観測できる分子デザインを施した。本来、三重項増感作用と蛍光放出とは同じ励起状態を中間体として経由するため、競合する。しかし分子デザインを工夫すれば両方の性質を満たす分子が創製できると考察した。本章では光増感剤としてフルオレノンを選択している。フルオレノンとフロキサンが一体化した分子を合成し性質を精査したところ、有機溶媒中では光増感は良好に進行しさらに分子は蛍光を発することを明らかにした。しかしながら、水溶液中においては光増感作用が消失し、その結果一酸化窒素放出は確認されなかった。この現象に対して、今回合成した分子は電荷移動型励起状態を形成するため、水のような極性溶媒中ではその励起状態が極度に安定化されてしまい無輻射放射が優先することを要因として提唱している。

　第四章では、第三章で観測できなかった水溶液中での一酸化窒素放出、蛍光放出を示す分子の創製について述べている。ナフタレンジイミドは優れた三重項増感作用と蛍光放出能を有することが文献調査で明らかとなった。そこで、ナフタレンジイミドをフロキサンと一体化する分子の創製を行った。合成した一体型分子は、光を照射すると水溶液中で一酸化窒素を放出することが分かった。光を照射しないと一酸化窒素は放出されないことから、本分子が光応答性一酸化窒素ドナーであることを明確に示した。また、本分子は水中で 581 nm を最大とする蛍光を発した。本分子を HeLa 細胞に散布したところ、この分子が細胞内に取り込まれることを蛍光顕微鏡にて観測することができたと述べている。

　第五章では、論文の総括および今後の展望が、第六章以降では実験の詳細がそれぞれ述べられている。

　以上のように、本論文では、フロキサンを基本骨格とする分子修飾を基にして、光応答性一酸化窒素ドナーに関する知見を収集した。有機合成の観点からは、フロキサンは種々の反応条件に不安定であり分子修飾は困難であるという認識を覆し、多くの新規な反応を開発した。光化学の観点からは、フロキサンの光増感作用を発見しそれを長波長励起可能な光応答性一酸化窒素ドナーの創製まで応用展開させた。本論文は、新しい光応答性一酸化窒素ドナーについての重要な知見を十分得たものとして価値ある集積である。よって、学位申請者の Christopher Peter Seymour は、博士（学術）の学位を得る資格があると認める。