"Dark" singlet oxygen and electron paramagnetic resonance spin trapping as convenient tools to assess photolytic drug degradation

"Dark" Singlet Oxygen and EPR Spin Trapping as Convenient Tools to Assess Photolytic Drug Degradation

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Abstract

Forced degradation studies are an important tool for a systematic assessment of decomposition pathways and identification of reactive sites in active pharmaceutical ingredients (APIs). Two methodologies have been combined in order to provide a deeper understanding of singlet-oxygen related degradation pathways of APIs under light irradiation. Firstly, we report that a "dark" singlet oxygen test enables the investigation of drug reactivity towards singlet oxygen independently of photolytic irradiation processes. Secondly, the photosensitizing properties of the API producing the singlet oxygen was proven and quantified by spin trapping and EPR analysis. A combination of these techniques is an interesting addition to the forced degradation portfolio as it can be used for a) revealing unexpected degradation pathways of APIs due to singlet oxygen, b) clarifying photolytic drug-drug interactions in fixed dose combinations, and c) synthesizing larger quantities of hardly accessible oxidative drug degradants.

Keywords:

chemical stability; degradation products; photolysis; photodegradation; oxidation

Introduction

Active pharmaceutical ingredients (APIs) can easily be exposed to light anytime during manufacturing, storage, and in-use conditions. Hence, a thorough evaluation of photolytic reactivity and degradation pathways at an early stage is a prerequisite for the development of a safe drug. An adequate and reliable shelf life as well as product-safety precautions and protective actions to be taken during manufacturing, packaging, transport and storage depend on those studies. Consequently, the assessment of photolytic drug stability is required by means of regulatory guidelines such as ICH Q1B.[1]

Since in most cases photolytic degradation takes place in the presence of oxygen, a large number of photodegradation processes is oxidative in nature. These reactions can generally be classified as either Type I or Type II reactions (Scheme 1).[2] In a simplified picture, the API gets converted from its ground state (S_0) to a singlet excited state (S_1) upon light irradiation with an appropriate wavelength. While relaxation by fluorescence or radiationless processes lead to de-excitation, intersystem crossing (ISC) allows the formation of a triplet state (T_1). In its excited states, the photosensitized API can subsequently degrade via free radical or redox reactions, classically labelled as Type I reactions. Alternatively, the API in its triplet state (T_1) can also interact with triplet ground state molecular oxygen ($^3O_2$) to generate

singlet oxygen ($^1O_2$) via energy transfer. The latter process liberates the API in its ground state (S_0) again. In this case, the API acts as a photosensitizer and its chemical structure stays unchanged throughout this process as these transitions represent only changes of electron configurations within the API. Degradation will only occur, when the API subsequently reacts with the generated singlet oxygen, a transformation referred to as a Type II reaction. Due to its particularly high reactivity, singlet oxygen can react with a broad range of organic scaffolds. Many of those, e.g. heterocycles undergoing cycloadditions, are often present in small molecules APIs.[3] Although many sources of singlet oxygen are known,[4] photosensitization by the API is the most relevant in the context of drug degradation. Parameters like concentration and solvent have a strong impact on experimentally measured quantum yields for formation of singlet oxygen,[5] but the fact that these values can be quite high for some APIs (e.g. 0.62 for tiaprofenic acid and 0.39 for ketoprofen[6]) further illustrates the importance of understanding singlet oxygen induced Type II degradation processes.

Attempts to apply light irradiation as a synthetic tool to prepare sufficient amounts of Type II degradant for further structure or toxicological evaluation are often significantly hampered by concurrent complex Type I degradation processes. One approach to overcome this issue includes the addition of very potent photosensitizers like e.g. rose bengal to boost the light induced singlet oxygen generation and hence favor Type II reactions.[7] Unfortunately this procedure does not always suppress alternative Type I degradation pathways. The use of irradiation-free generated singlet oxygen - so called “dark” singlet oxygen - represents a valuable approach in order to synthesize the targeted degradant.

For such an irradiation-free procedure, different chemical methods to generate singlet oxygen can be considered. Classical approaches based on reagents like hypochlorite,[8] ozone,[9] or periodate,[10] are mainly limited to aqueous systems and are considered to be too harsh to be applicable for selective drug degradation processes. The use of a sodium molybdate/hydrogen peroxide microemulsion system of water in dichloromethane together with a surfactant and *n*-butanol appears to be a reasonable solution to circumvent these drawbacks.[11,12] In such a system, singlet oxygen is produced by reaction of hydrogen peroxide and sodium molybdate within aqueous micelles. After butanol-induced diffusion into the organic phase, singlet oxygen can then react with the API. The advantage of such a system is that the presence of peroxides is mainly restricted to the aqueous micelles, making the protocol applicable to APIs that are sensitive to peroxides.

As the corresponding analytical tool, electron paramagnetic resonance (EPR) in combination with spin trapping has been shown to be a useful technique to follow singlet oxygen generation through photosensitization.[13] When singlet oxygen is formed in the presence of the spin trap 4-hydroxy-2,2,6,6-tetramethylpiperidine (4-OH-TMP), a persistent TEMPOL-radical is formed, which can easily be detected by EPR-spectroscopy (Scheme 2).[14] It has been shown that 4-OH-TMP is a superior reagent compared to other sterically hindered amines for this purpose in terms of specificity and selectivity.[15] Since TEMPOL is a stable and commercially available compound, it can be used as a calibration standard, allowing quantification of the signal.

To our knowledge, by combining the two above-mentioned protocols, it is the first time that the photosensitizing potency of an API and its singlet oxygen induced degradation can be studied independently from each other. First, drug candidate TMC647055[16] was used to exemplify the general suitability of this methodology. Subsequently, our protocol was used to reveal photolytic incompatibility of two APIs - VU0409551/JNJ-46778212-AAA[17] and JNJ-54119936-AAA[18]. By means of the latter model study it was shown that the investigation of photolytic API-API interactions in fixed-dose combinations (FDC) is a useful application of this two-fold approach. Despite the rising importance of FDCs, in the vast number of studies stress testing is conducted on single compounds and examples using mixtures of APIs are scarce.[19] That disregard can be explained by the lack of uniform guidelines,[20] but also by the fact that in most cases the single components of an FDC have already been marketed as monotherapies and their individual degradation pathways are already well studied. The herein described procedure to separately address photosensitizing potency (by EPR spin trapping) and reactivity towards singlet oxygen (by dark singlet oxygen) conveniently allows the identification of API couples in which one is a potent photosensitizer while the other one is very sensitive towards reaction with singlet oxygen. This approach allows foreseeing potential API-API incompatibilities already by performing stress testing of the single components only.

Experimental

Material: TMC647055 (**1**), VU0409551/JNJ-46778212-AAA (**4**), and JNJ-54119936-AAA (**5**) were obtained from Janssen Pharmaceutica N.V. Other reagents were obtained from Sigma-Aldrich and used without further purification.

Light Irradiation: For light irradiation each compound (VU0409551/JNJ-46778212-AAA (**4**) or JNJ-54119936-AAA (**5**)) was dissolved in a mixture of THF/H_2O (1:1) to give a final concentration of 5 mM. For the detection of singlet oxygen, spin-trap 4-hydroxy-2,2,6,6-tetramethylpiperidine (4-OH-TMP, *c* = 100 mM) was also added. Solutions were placed in 10 mL screw cap vials (quartz) and irradiated at 300 W/m^2 at a constant ambient temperature in a Suntest CPS+ apparatus equipped with a xenon lamp filtered with window glass (6 mm) and a solar ID65 filter.

Electron paramagnetic resonance (EPR) spin trapping: Electron paramagnetic resonance measurements were performed with an X-band EPR-spectrometer (Bruker e-scan). For the detection of 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxyl (TEMPOL) after irradiation, the solution was filled into a 50 µL Blaubrand® micropipette, sealed, and placed into a capillary holder to be analyzed by EPR-spectroscopy. Instrument settings were: microwave power, 2.15 mW; microwave frequency 9.73 GHz; field sweep, 80 G; modulation frequency 86.0 kHz; modulation amplitude 0.99 G; sweep time, 10.49 sec; time constant; 1.25 msec. Commercially available TEMPOL was used as a calibration standard for the determination of spin concentrations after double integration of the obtained signals. The limit of quantitation was calculated to be 4.5 µM (see supporting information).

Dark Singlet Oxygen Testing: A solution of sodium molybdate (1.15 g, 5.58 mmol) in water (6 mL) was added dropwise to a stirred suspension of sodium dodecylsulfate (9.5 g, 32.9 mmol) in *n*-butanol (9.5 g) and dichloromethane (9.5 g) until a clear solution was obtained.

- TMC647055 (**1**) (182 mg, 0.3 mmol) was dissolved in 6.5 mL of the prepared sodium molybdate solution and stirred in the dark. In the course of six hours, 13 portions of 30% aqueous hydrogen peroxide solution (40 µL) were added. The reaction progress was monitored by HPLC. Once the conversion was complete, 10 mL dichloromethane and 10 mL of a saturated aqueous NaCl solution were added to the reaction mixture. The two layers were separated and the aqueous phase was extracted three additional times with dichloromethane. The organic layers were combined, dried with Na_2SO_4, filtered and evaporated under reduced pressure. After purification by flash column chromatography (Eluent: dichloromethane /MeOH), **3** was obtained as a beige solid with a yield of 65% (125 mg, 0.19 mmol). ^{1}H NMR (DMSO-d_6, 601MHz, 380K) δ = 7.88 (d, *J*=8.7 Hz, 1H), 7.86 (dd, *J*=1.5, 7.9 Hz, 1H), 7.73 (d, *J*=7.9 Hz, 1H), 7.72 (d, *J*=1.4 Hz, 1H), 7.18 (s, 1H), 7.12 (dd, *J*=2.6, 8.7 Hz, 1H), 7.04 (d, *J*=2.4 Hz, 1H), 4.09 (s, 2H), 3.90 (s, 3H), 3.67 (t, *J*=5.4 Hz, 2H), 3.63 (t, *J*=5.4 Hz, 2H), 3.56 (t, *J*=5.4 Hz, 2H), 3.51 (t, *J*=5.4 Hz, 2H), 3.22 (s, 3H), 3.10 (s, 3H), 3.13 - 3.05 (m, 1H), 1.94 - 1.86 (m, 2H), 1.81 - 1.74 (m, 2H), 1.70 - 1.62 (m, 1H), 1.50 - 1.40 (m, 2H), 1.40 - 1.31 (m, 2H), 1.30 - 1.23 (m, 1H) ppm; ^{13}C NMR (DMSO-d_6, 151MHz , 380K): δ = 204.3, 204.3, 168.8, 168.0, 166.6, 166.5, 166.2, 163.8, 160.8, 140.5, 140.2, 140.1, 139.0, 136.8, 136.5, 135.4, 135.3, 134.8, 133.0, 132.4, 132.1, 127.6, 127.4, 127.1, 126.5, 126.4, 126.3, 125.3, 114.8, 114.5, 113.5, 68.6, 67.5, 67.0, 64.9, 55.0, 55.0, 49.7, 49.3, 48.9, 47.8, 47.6 (br s), 47.5, 47.3, 36.1, 35.1 (br s), 32.5 (br s), 28.0, 27.9, 24.8, 24.8, 24.4, 24.4 ppm. HRMS: calcd. for $M+H^+$ ($C_{32}H_{39}N_4O_8S$): 639.2489 found: 639.2480.
- To assess the reactivity of VU0409551/JNJ-46778212-AAA (**4**) and JNJ-54119936-AAA (**5**), the corresponding API (0.06 mmol; 21.1 mg for **4**; 34.9 mg for **5**) was dissolved in 1 mL of the prepared sodium molybdate solution and stirred in the dark. In the course of three hours, five portions of 30% aqueous hydrogen peroxide solution (10 µL) were added. The reaction progress was monitored by HPLC (50 µL of the mixture, diluting with water/acetonitrile (1:1, 5 mL)).
- 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoic acid (**10**) (181.89 mg, 0.3 mmol) was dissolved in 6.5 mL of the prepared sodium molybdate solution and stirred in the dark. In the course of six hours, 11 potions of 30% aqueous hydrogen peroxide solution (40 µL) were added. The reaction progress was monitored by HPLC and reaction product **11** characterized by mass spectrometry. For further experimental details see the supporting information.

Results and Discussion

Degradation of TMC647055 (**1**)

During forced degradation studies, drug candidate TMC647055 (**1**) was found to be sensitive towards irradiation with light in solution or as a solid (Scheme 3). In both cases, amide **3** turned out to be one of the major photolytic degradants. A reasonable degradation route towards **3** would be a [2+2] cycloaddition of singlet oxygen to C2-C3 of the indole moiety of **1** to give a four membered dioxetane intermediate **2**, followed by an electrocyclic ring opening yielding the amide **3**.

Unfortunately, irradiation of **1** with simulated daylight filtered through window glass (300 W/m^2) as a solid or in solution turned out to be inefficient processes in order to synthesize **3**. While as a solid barely any reaction happened; in solution a complex mixture was obtained rendering purification not easy

(Scheme 4a). Also, irradiation of **1** in the presence of rose bengal gave only unclean conversion towards **3** (Scheme 4a). In contrast, when exposed to the conditions of dark singlet oxygen described above, a clean formation of the degradant **3** is observed (Scheme 4b). To prove the applicability of the protocol on a semi preparative scale, the reaction was performed on 125 mg of the API. After purification of the crude reaction mixture, amide **3** was obtained in 65% yield.

In addition, further confirmation of the involvement of singlet oxygen in the photolytic degradation process was obtained by conducting EPR spin trapping experiments. When irradiating a solution of **1** in THF/water in the presence of 4-OH-TMP for two hours, TEMPOL formation was detected with a concentration of 15 µM (Table 1; entry 1). Higher levels of TEMPOL (22 µM) (entry 2) were observed when the irradiation was performed in a D_2O/THF mixture. This represents a further evidence for singlet oxygen being the active reactive oxygen species in this case, as D_2O is known to increase the lifetime of singlet oxygen in solution.[21] Longer irradiation time leads to higher TEMPOL concentration as well (42 µM after four hours) (entry 3). While singlet oxygen quencher sodium azide entirely prevents the formation of TEMPOL (entry 4), the addition of hydroxyl radical quencher mannitol had as expected almost no impact on TEMPOL concentrations (13 µM) (entry 5). Although in some cases indicative of other oxidation pathways as well,[22] the EPR spin trapping analysis experimental setup was specific towards the detection of singlet oxygen: neither the addition of hydrogen peroxide (entry 6) nor a Fenton[23] like system (entry 7) as source of hydroxyl radicals gave a detectable TEMPOL signal. The occurrence of a TEMPOL background signal is observed only after a prolonged irradiation time of more than three hours or storage of an irradiated solution for several days (data not shown).

This EPR spin trapping protocol turned out to be robust, reliable and applicable to a broad range of APIs. In a benchmarking study conducted on 56 different APIs, 25% of them turned out to act as photosensitizers when irradiated for one hour: six APIs generated TEMPOL in the range of 10-20 µM, four APIs gave values of 20-100 µM, two APIs gave values of 100-200 µM and one API exceeded the value of 200 µM TEMPOL.

This nicely illustrates the importance to consider and evaluate singlet oxygen related degradation routes in drug development. However, only the situation in solution is covered with this particular experimental setup. Despite the fact that the estimated diffusion length of singlet oxygen in air (2.7 mm) should be sufficient to enable a reaction in solids as well, this scenario likely differs from that in solution.[3]

Photolytic API-API interaction

In a model study, we studied the behavior of two APIs as solutions under photolytic conditions either alone or in combination. The two APIs - VU0409551/JNJ-46778212-AAA (**4**) and JNJ-54119936-AAA (**5**) - are currently being independently developed as drug candidates (Scheme 5). Although **4** and **5** are not considered to be used as a FDC, this study serves as a model case to illustrate unexpected chemical stability features of FDCs in comparison to the chemical behavior of single components under photolytic conditions.

When irradiated separately with simulated daylight filtered through window glass (300 W/m^2), compounds **4** and **5** both turned out to be reasonably stable. Only after a very long irradiation time (8

hours) a slow decay of the active ingredients was observed (Scheme 6a,b). Also when irradiated as solids or suspension in water, practically no degradation was observed (not shown). Based on these findings, no - or at least only very little - stability issues upon light irradiation would be expected.

Surprisingly, a solution in which **4** and **5** are combined in the same vial, complete degradation of **4** is observed after eight hours of irradiation (Scheme 6c). This degradation is obviously impossible to predict on the basis of single compound stress testing alone. Although unexpected at first sight, the underlying chemical process can be well explained with the photosensitizing ability of **5**. According to the general concept (Scheme 1), it is assumed that upon light irradiation and photon absorption, **5** is transferred from singlet ground state (S_0) to an excited singlet state (S_1) which in an intersystem crossing (ISC) is converted to a triplet state (T_1). Subsequently, a reaction with naturally occurring triplet oxygen ($^3O_2$) leads to the formation of singlet oxygen ($^1O_2$) and liberation of **5** in its singlet ground state again (Scheme 7, left).

Compound **5** is not consumed while passing through that cycle and continuously generates singlet oxygen while being stable towards it. In other words, as long as its different electronic states are stable and do not decompose in a Type I reaction or as long as it is not undergoing a Type II reaction, **5** can be regarded as an organocatalyst being overall stable under light exposure. In contrast to **5**, **4** is particularly reactive towards singlet oxygen and decomposes readily following a [4+2] cycloaddition at the oxazole moiety (Scheme 7, right). The generated endoperoxide **6** is prone to immediate opening and formation of anhydride **7**. Finally, hydrolysis of anhydride **7** gives main degradants **8** and **9**. Since **4** is not a photosensitizer itself, no such degradation can be observed when it is irradiated as single compound. Hence, this potential degradation pathway in a FDC would not be predicted.

APIs **4** and **5** were separately exposed to light irradiation in the presence of spin trap 4-OH-TMP while the amount of TEMPOL formed was regularly monitored. Only **5** caused increasing levels of TEMPOL, proving it to act as the photosensitizer generating singlet oxygen. Even after a prolonged irradiation time, **4** only gave a signal not exceeding the background process (Scheme 8).

Subsequently, both APIs were separately exposed to the dark singlet oxygen conditions and the reaction progress was regularly monitored by HPLC. As expected, we observed that **4** indeed reacts readily to give mainly degradants **8** and **9**, while **5** remains entirely untouched (Scheme 9).

The high reactivity of **4** as well as the particular stability of **5** towards singlet oxygen are consistent with the assumed degradation pathway. The fact that numerous heterocycles - being known to react readily with singlet oxygen - are widely used in APIs[24] taken together with our benchmarking study showing 25% of APIs to be photosensitizers, illustrates that this API – API incompatibility scenario is not an unusual coincidence.

Dark Singlet Oxygen as selective oxidant

The dark singlet oxygen approach was successfully applied as a suitable tool to prove the formation of oxidative degradants of excipients in a complex drug product formulation. Upon thermal stability testing, an unknown peak was observed in the HPLC chromatogram of a liquid injectable solution. This

compound was present in amounts that did not suffice for isolation and further structural elucidation, but the UV profile indicated an origin different from the API. To verify the hypothesis of an oxidative degradation process, single components present in the drug product as excipients were exposed to dark singlet oxygen conditions and the reaction products were compared to the unknown compound. It turned out that BHT analog and antioxidant 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propanoic acid (**10**) gave a reaction product that showed a perfect conformity in terms of retention time, UV profile and MS spectrum with the unknown degradant when exposed to dark singlet oxygen.

A further analysis of the MS spectrum and the corresponding fragmentation pattern lead to the conclusion this degradant to be hydroperoxide **11** (see supporting information). Since phenols like **10** are prone to a keto-enole tautomerism, a ring double bond can participate in an Alder-ene type reaction with singlet oxygen to give hydroperoxide **11**. Although alternative oxidative approaches for alkyl phenols are known,[25] the dark singlet oxygen methodology enables the formation of **11** under mild conditions in a very selective manner. The excellent conversion of **10** to give **11** as the sole reaction product with an *in situ* yield of 92% (Scheme 10) further illustrates the quality of the dark singlet oxygen methodology as a synthetic tool to generate large quantities of various oxidative degradants.

Conclusions

The development of novel and reliable stability testing protocols is important to assess stability issues of drugs as early as possible and to avoid unexpected degradation. The described dark singlet oxygen stress test in combination with EPR spin trapping as the corresponding analytical tool is a user friendly approach to study singlet oxygen related drug degradation processes. Due to its specificity, selectivity, and mild reaction conditions, the dark singlet oxygen protocol is complementary to more classical irradiation based approaches such as the addition of rose bengal or other photosensitizers and has successfully been applied on different compounds like drug candidates and excipients. Beyond its application in stress testing, this procedure can also be used for the synthetic preparation of oxidative degradants.

On the other hand, EPR spin trapping was used to review the photosensitizing power of 56 APIs. This methodology is not intended for accurate quantifications of quantum yields, but rather to conveniently estimate the photosensitization abilities of APIs being sufficient for the needs in pharmaceutical stress testing and for understanding the mechanistic degradation pathways towards the observed degradants.

This two-tiered procedure can easily be implemented as part of a mostly standardized portfolio of stress testing experiments. Even mechanistically complex and otherwise barely predictable degradation processes like those based on photolytic drug-drug or drug-excipient interactions can be revealed.

Acknowledgments

We thank Vanderbilt University for the permission to publish our results on VU0409551/JNJ-46778212-AAA.

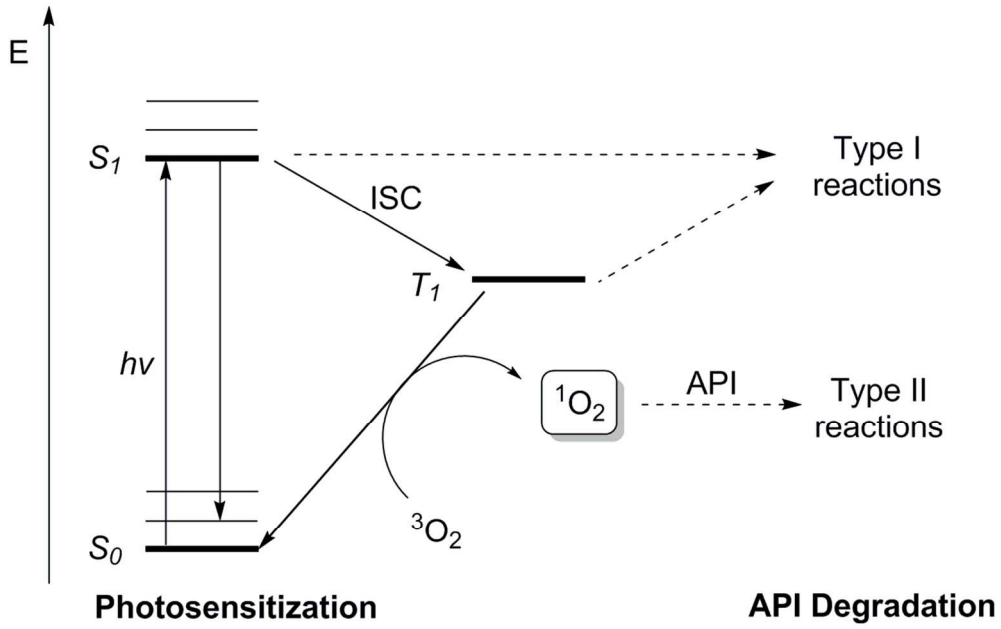
The authors declare no competing financial interest.

[1] ICH Q1B. 1997. Photostability testing of new drug substances and products. Fed Reg 62: 27115–27122.
[2] Li M. 2012. Photochemical Degradation. Organic Chemistry of Drug Degradation, 1st ed., Cambridge: The Royal Society of Chemistry p 165-197.
[3] Clennan EL, Pace A. 2005. Advances in singlet oxygen chemistry. Tetrahedron 61: 6665-6691.
[4] Schweitzer C, Schmidt R. 2003. Physical Mechanisms of Generation and Deactivation of Singlet Oxygen. Chem Rev 103: 1685-1757.
[5] Wilkinson F, Helman WP, Ross AB. 1993. Quantum Yields for the Photosensitized Formation of the Lowest Electronically Excited State of molecular Oxygen in Solution. J Phys Chem Ref Data 22: 113-262.
[6] Bosca F, Marin ML, Miranda MA. 2001. Photoreactivity of the Nonsteroidal Anti-inflammatory 2-Arylpropionic Acids with Photosensitizing Side Effects. Photochem Photobiol 74: 637-355.
[7] Vargas F, Hisbeth MV, Rojas JK. 1998. Photolysis and photosensitized degradation of the diuretic drug acetazolamide. J Photochem Photobiol A 118: 19-23.
[8] Held AM, Halko DJ, Hurst JK. 1978. Mechanisms of Chlorine Oxidation of Hydrogen Peroxide. J Am Chem Soc 100: 5732-5740.
[9] Wasserman HH, Yoo JU, DeSimone RW. 1995. Singlet Oxygen Reactions from the Adducts of Ozone with Heterocyclic Substrates. J Am Chem Soc 117: 9772-9773.
[10] Bokare AD, Choi W. 2015. Singlet-oxygen Generation in Alkaline Periodate Solution. Environ Sci Technol 49: 14392-14400.
[11] Aubry JM, Bouttemy S. 1997. Preparative Oxidation of Organic Compounds in Microemulsions with Singlet Oxygen generated Chemically by Sodium Molybdate/Hydrogen Peroxide System. J Am Chem Soc 119: 5286-5294.
[12] Aubry JM, Adam W, Alsters PL, Borde C, Queste S, Marko J, Nardello V. 2006. Dark singlet oxygenation of organic substrates in single-phase and multiphase microemulsion systems. Tetrahedron 62: 10753-10761.
[13] Moan J, Wold E. 1979. Detection of singlet oxygen production by ESR. Nature 279: 450-451.
[14] Lion Y, Gandin E, Van De Vorst A. 1980. On the Production of Nitroxide Radicals by Singlet Oxygen reaction: An EPR Study. Photochem Photobiol 31: 305-309.
[15] Nakamura K, Ishiyama K, Ikai H, Kanno T, Sasaki K, Niwano Y, Kohno M. 2011. Reevaluation of analytical methods for photogenerated singlet oxygen. J Clin Biochem Nutr 49:87-95.
[16] Cummings MD, Lin TI, Hu L, Tahri A, McGowan D, Amssoms K, Last S, Devogelaere B, Rouan MC, Vijgen L, Berke JM, Dehertogh P, Fransen E, Cleiren E, van der Helm L, Fanning G, Nyanguile O, Simmen K, Van Remoortere P, Raboisson P, Vendeville S. 2013. Discovery and Early Development of TMC647055, a Non-Nucleoside Inhibitor of the Hepatitis C Virus NS5B Polymerase. J Med Chem 57: 1880-1892.
[17] Conn PJ, Lindsley CW, Stauffer SR, Jones CK, Bartolome-Nebreda JM, Conde-Ceide S, Macdonald GJ, Alcazar, WO 2012/031024 A1.
[18] Leonard KA, Barbay K, Edwards JP, Kreutter KD, Kummer DA, Maharoof U, Nishimura R, Urbanski M, Venkatesan H, Wang A, Wolin RL, Woods CR, Pierce J, Goldberg S, Fourie A, Xue X. US 2014/0107094 A1
[19] Reynolds DW, Joshi BK. 2011. Stress testing of combination therapies. In Baertschi SW, Alsante KM, Reed RA, editors. Pharmaceutical stress testing – Predicting drug degradation, 2nd ed., London: Informa Healthcare p 447-459.
[20] WHO Technical Report Series 929. Geneva, 2005. WHO Expert Committee on Specifications for Pharmaceutical Preparations.
[21] Wilkinson F, Helman WP, Ross AB. 1995. Rate Constants for the Decay and Reactions of the Lowest Electronically Excited Singlet State of Molecular Oxygen in Solution. An Expanded and Revised Compilation. J Phys Chem Ref Data 24: 663-1021.
[22] Nardi G, Manet I, Monti S, Miranda MA, Lhiaubet-Vallet V. 2014. Scope and limitations of the TEMPO/EPR method for singlet oxygen detection: the misleading role of electron transfer. Free Rad Biol Med 77: 64-70.
[23] Mizuta Y, Masumizu T, Kohno M, Mori A, Packer L. 1997. Kinetic analysis of the Fenton reaction by ESR-spin trapping. Biochem Mol Biol Int 43: 1107-1120.
[24] Li JJ (edit.). 2013. Heterocyclic Chemistry in Drug Discovery, 1st ed., Hoboken: John Wiley and Sons.

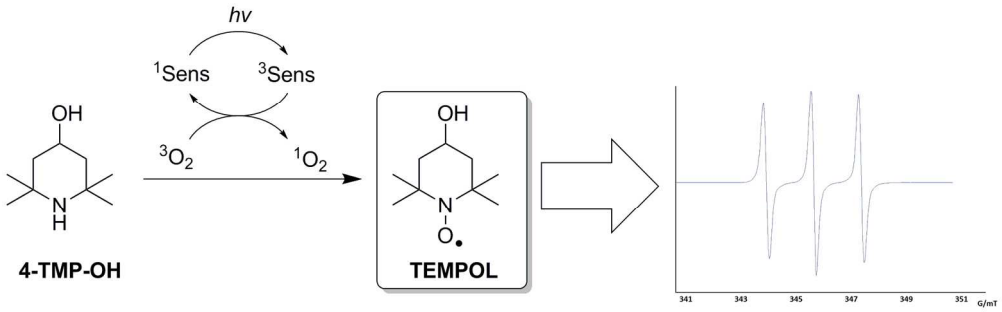
[25] Carreno MC, Gonzales-Lopez M, Urbano A. 2006. Oxidative De-aromatization of para-Alkyl Phenols into para-Peroxyquinols and para-Quinols Mediated by Oxone as a Source of Singlet Oxygen. Angew Chem Int Ed 45: 2737-2741.

Table 1: Formation of TEMPOL upon light irradiation to evaluate photosensitizing potency of **1**

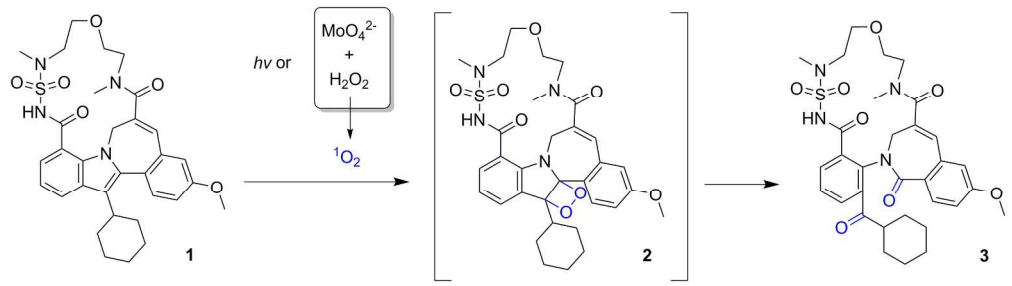
entry	solvent	Additive	Irradiation time	conc. TEMPOL [µM]
1	H_2O/THF (1:1)	---	2h	15
2	D_2O/THF (1:1)	---	2h	22
3	H_2O/THF (1:1)	---	4h	42
4	H_2O/THF (1:1)	NaN_3 (100 mM)	2h	not detected
5	H_2O/THF (1:1)	mannitol (100 mM)	2h	13
6	H_2O/THF (1:1)	H_2O_2 (1 mM)	2h (storage without irradiation)	not detected
7	H_2O/THF (1:1)	H_2O_2 (500 µM) + $FeSO_4$ (5 µM)	2h (storage without irradiation)	not detected



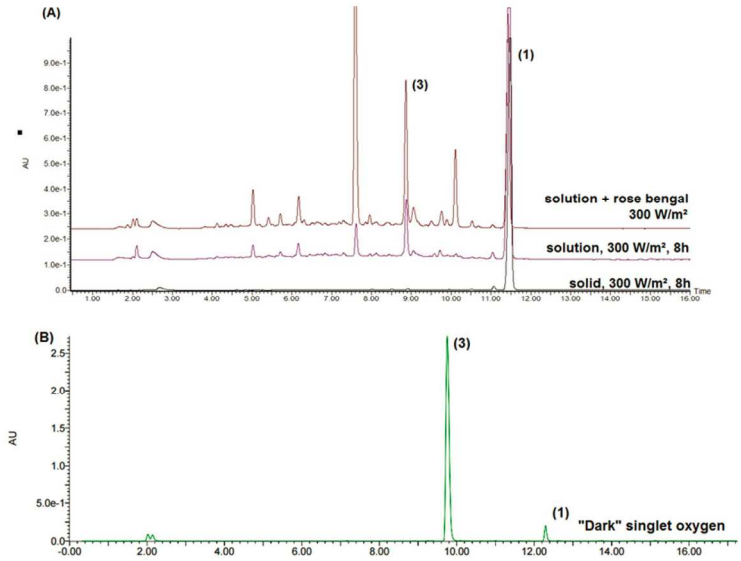
Scheme 1: Photolytic drug degradation (simplified)
Scheme 1
110x70mm (300 x 300 DPI)



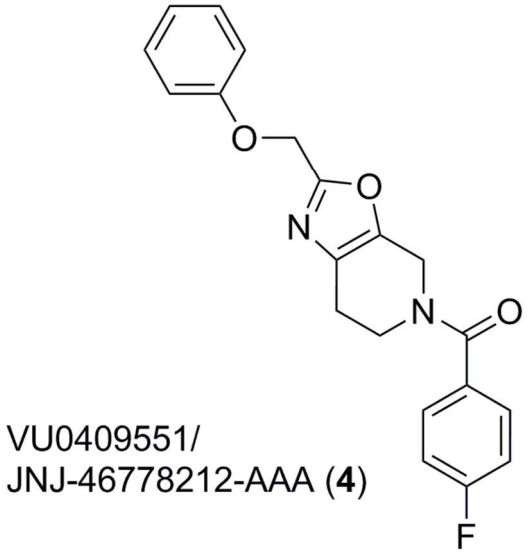
Scheme 2: Formation of TEMPOL by a reaction of 4-OH-TMP with singlet oxygen and its EPR spectrum.
Scheme 2
166x52mm (300 x 300 DPI)



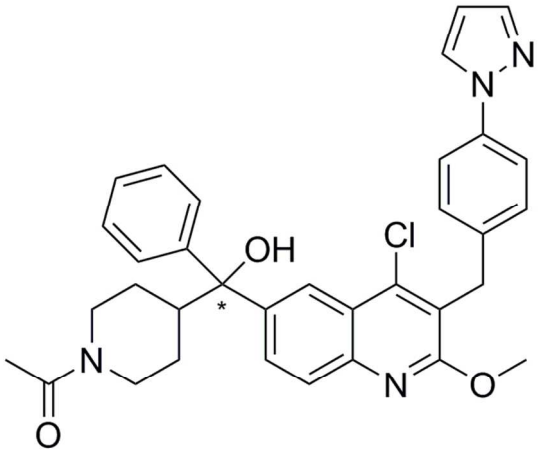
Scheme 3: Degradation of TMC647055 (1) upon light irradiation or in a dark singlet oxygen stress test.
Scheme 3
208x58mm (300 x 300 DPI)



Scheme 4: Chromatograms of TMC647055 (1) after light irradiation or exposure to dark singlet oxygen.
Scheme 4
279x215mm (300 x 300 DPI)



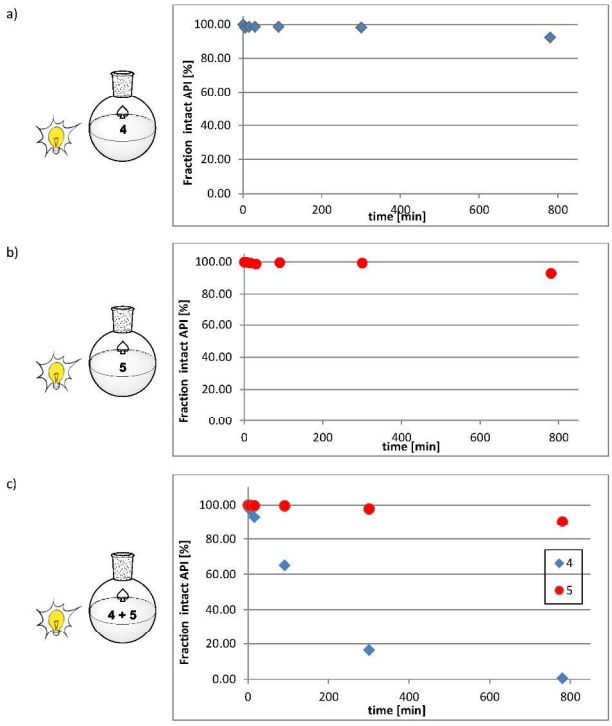
VU0409551/
JNJ-46778212-AAA (**4**)



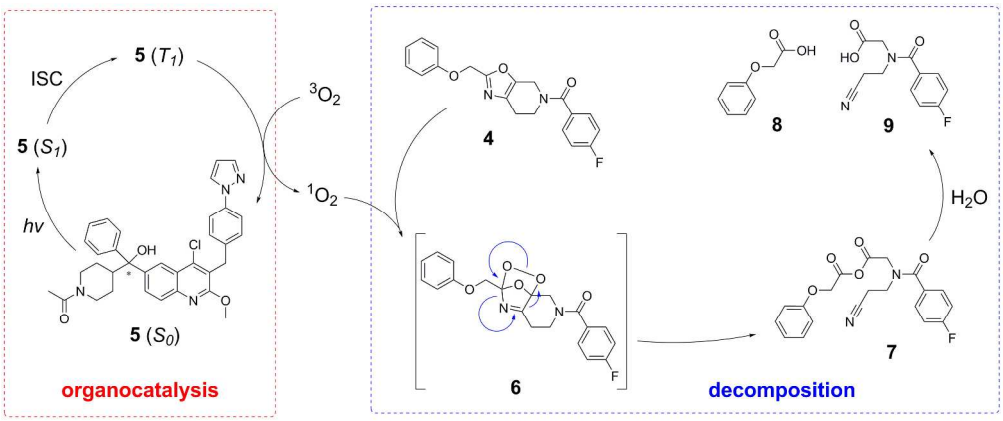
JNJ-54119936-AAA (**5**)

Scheme 5: Active pharmaceutical ingredients VU0409551/JNJ-46778212-AAA (4) and JNJ-54119936-AAA (5).

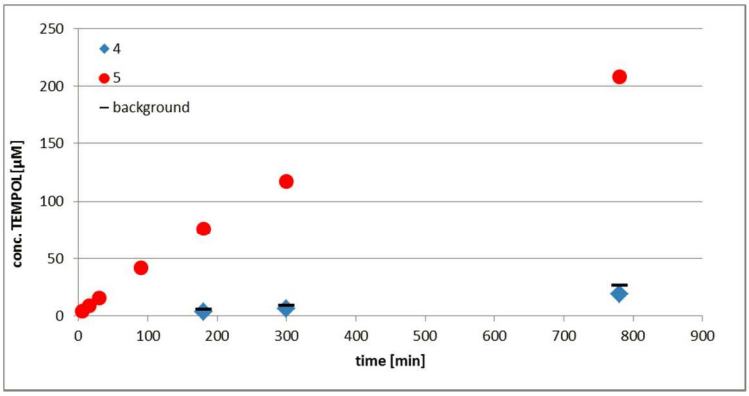
Scheme 5
55x121mm (300 x 300 DPI)



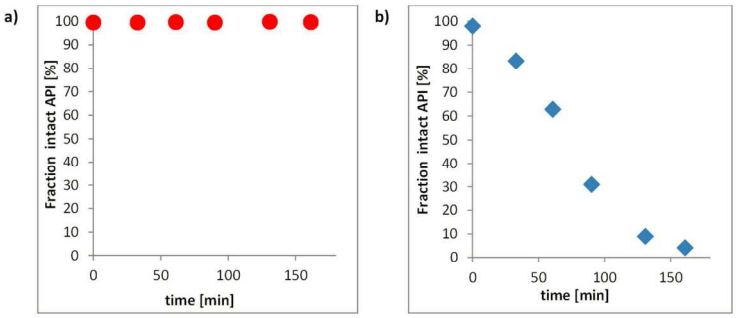
Scheme 6: Light induced degradation of 4 and 5 either in separate vials (a) and (b) or in combination (c).
Scheme 6
254x190mm (300 x 300 DPI)



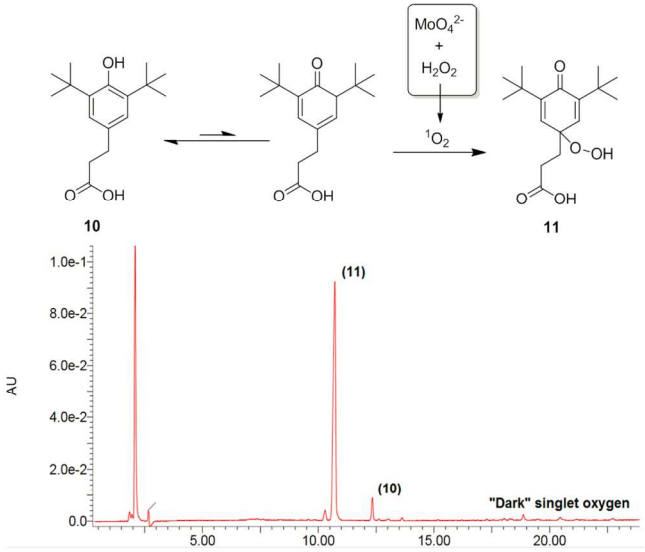
Scheme 7: 5 serving as a catalyst in the photolytic decomposition process of 4.
Scheme 7
272x113mm (300 x 300 DPI)



Scheme 8: Time dependent formation of TEMPOL upon irradiation of solutions containing 5 (red) or 4 (blue) in comparison to the background reaction of TEMPOL-formation (black).
Scheme 8
279x215mm (300 x 300 DPI)



Scheme 9: Stability of 5 (a) and 4 (b) in a dark singlet oxygen stress test.
Scheme 9
279x215mm (300 x 300 DPI)

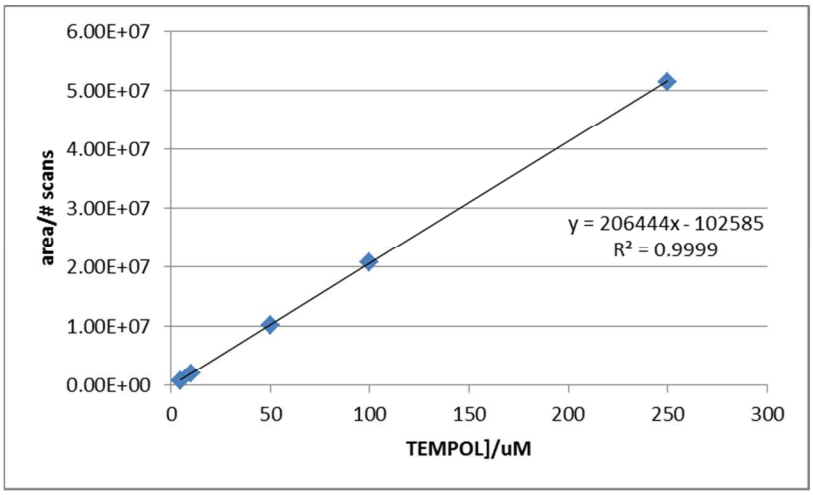


Scheme 10: Preparation of oxidative degradant 11 from antioxidant 10 together with its chromatogram.
Scheme 10
279x215mm (300 x 300 DPI)

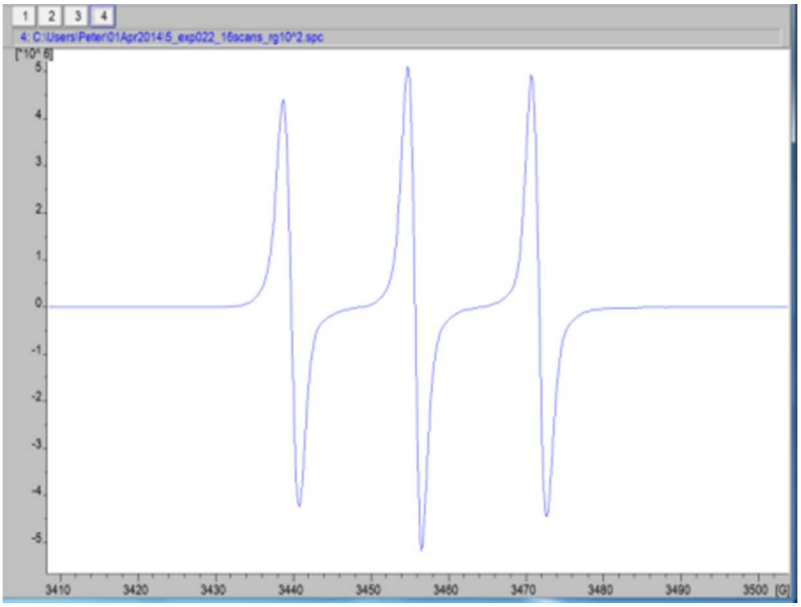
Supporting Information

Electron Paramagnetic Resonance - Spin Trapping:

In order to quantify the amount of TEMPOL by EPR, linearity of test method was verified. Solutions of 1000, 800, 400, 200, 100, 80, 40, 20, 10 µM TEMPOL in water were prepared using material from a commercial source (Sigma Aldrich). Concentrations were nominal and not corrected. Solutions were filled into a 50 µL Blaubrand® micropipette, sealed, and placed into a capillary holder to be analyzed by EPR-spectroscopy. Instrument settings were: microwave power, 2.15 mW; microwave frequency 9.73 GHz; field sweep, 80 G; modulation frequency 86.0 kHz; modulation amplitude 0.99 G; sweep time, 10.49 sec; time constant; 1.25 msec. Data were processed by double integration of obtained signals and divided by number of scans. The limit of detection was calculated to be 1.4 µM (3*standard deviation/slope) and the limit of quantitation was calculated to be 4.5 µM (10*standard deviation/slope).



Representative spectrum of TEMPOL:

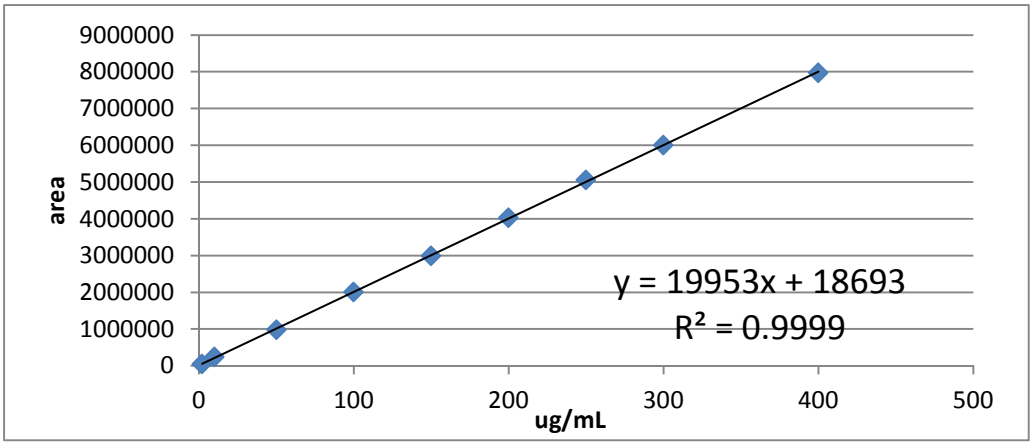


HPLC analysis of mGlu5PAM (**4**), and RORγt (**5**)

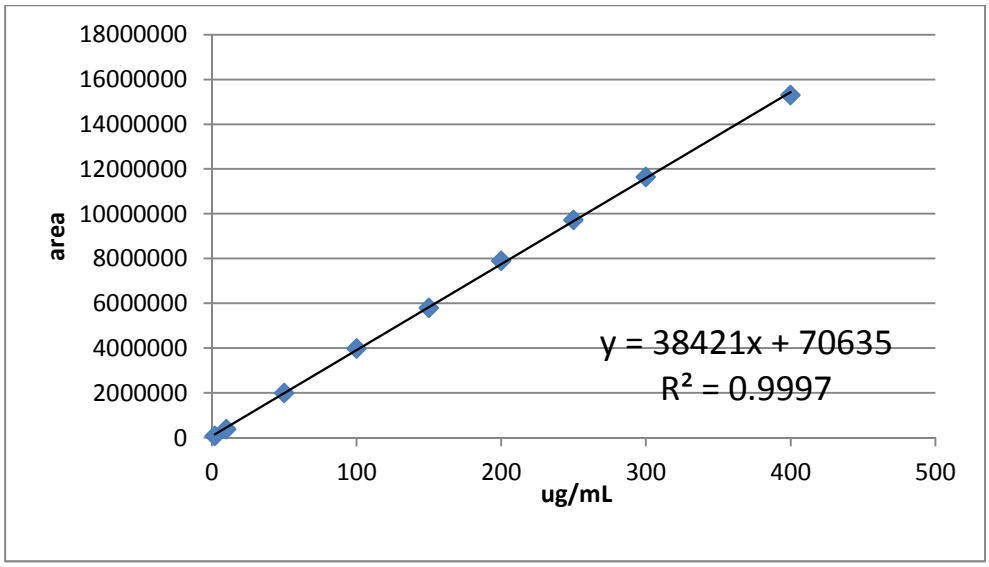
HPLC analyses of mGlu5PAM (**4**), and RORγt (**5**) were performed on a Waters HPLC system equipped with a diode array detector. The used 4.6x150 mm X-Bridge C18 column, 3.5 µM (Waters) was operated at 30°C with a constant flow of 0.8 mL/min. The mobile phases consisted of acetonitrile (A) and a 10 mM aqueous NH_4OAc solutions (B). Phase A was increased linearly from 5 % (v/v) to 100 % (v/v) over 20 min and held at 100% for 5 min. Data were processed at 242 nm for RORγt, and 222 nm for mGlu5PAM. Values are calculated as peak area relative to peak area measured before irradiation as a mean of five measurements. In order to quantify the amount of mGlu 5 PAM and RORγt MQM by HPLC, linearity of test method was verified.

Eluent A: acetonitrile; eluent B: 10 mM ammonium acetate in water; injection volume: 5 µL, flow: 0.8 mL/min; column: X-Bridge C18, 3.5 µM, 4.6x150 mm; gradient: 5% to 100% (v/v) A, 20 min linear gradient, then constant for 5 min.

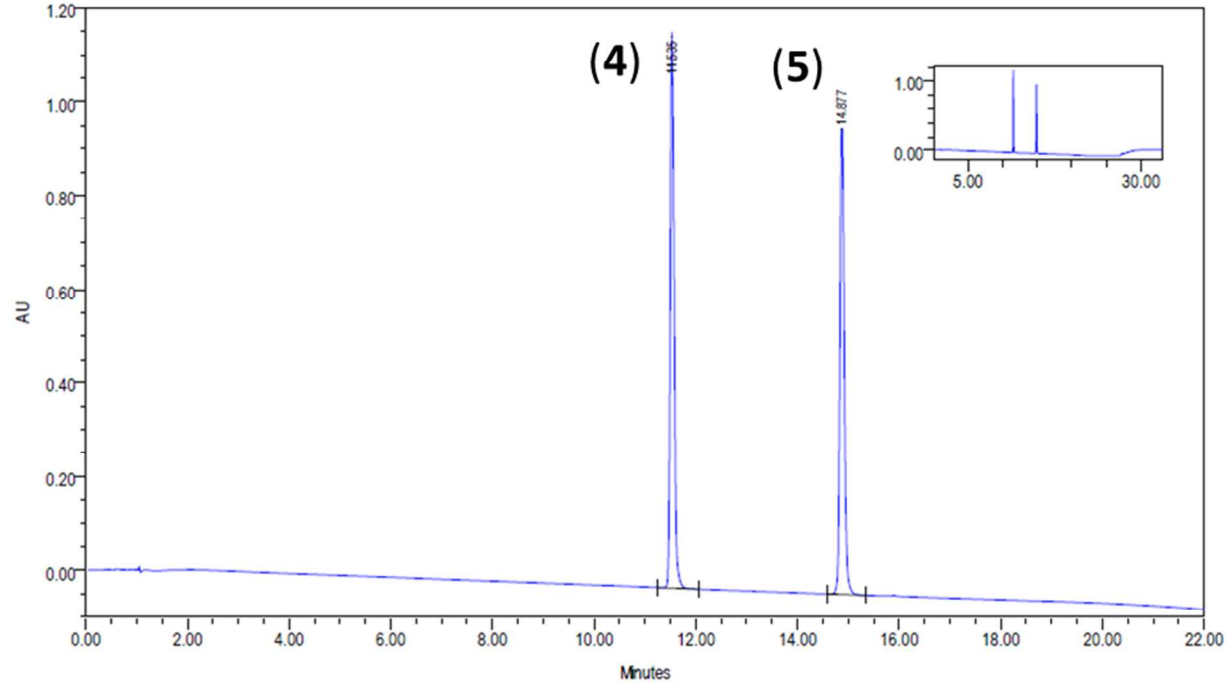
Solutions of 400, 300, 250, 200, 150, 100, 50, 10, 2 µg/mL mGlu5PAM in acetonitrile were prepared using a reference standard and processed at 222 nm.



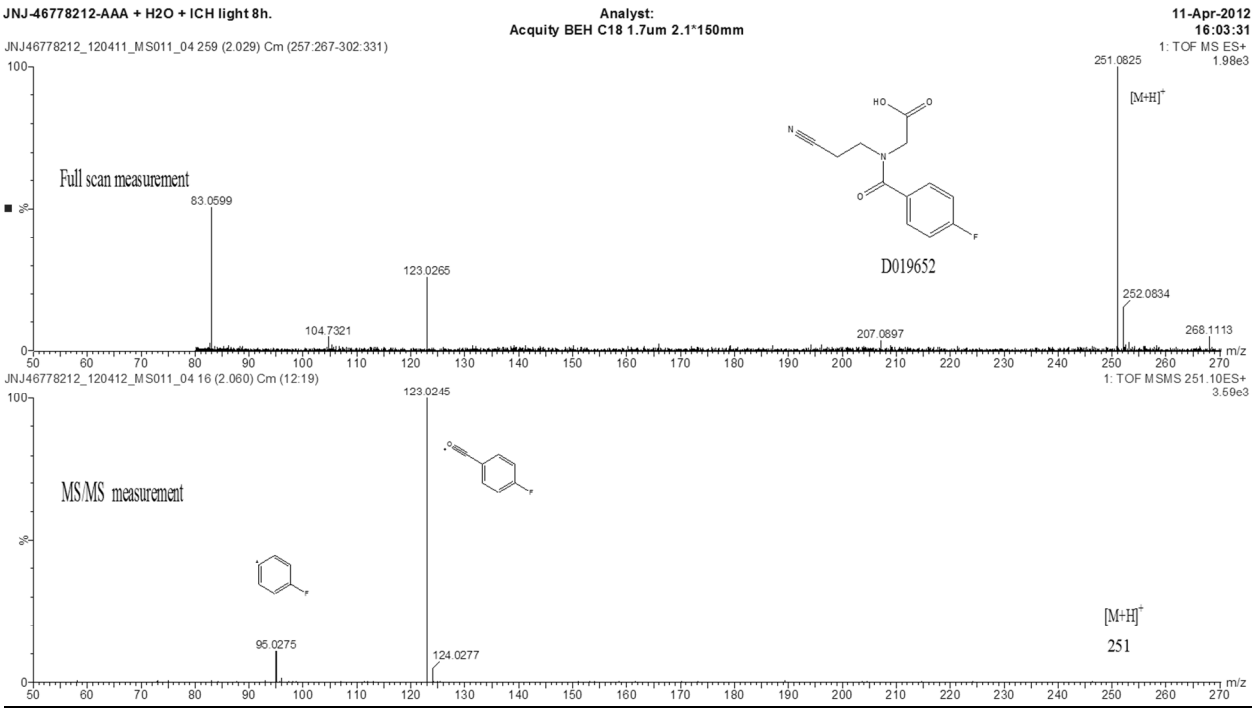
Solutions of 400, 300, 250, 200, 150, 100, 50, 20, 10, 2 µg/mL RORγt in acetonitrile were prepared using a reference standard and processed at 242 nm.



Representative chromatogram of binary mixture (5 mM each):

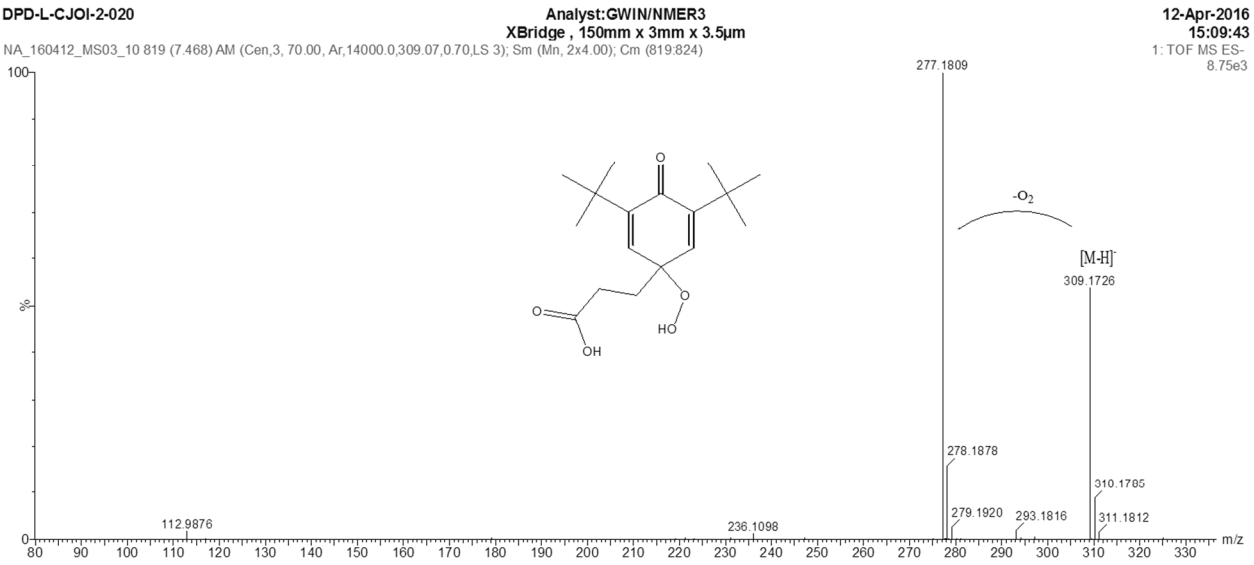


Mass spectrometric analysis of reaction product **9**:



LC-MS and LC-MS-MS analyses of D 019652 (**9**) were performed on a Waters UPLC system coupled to a Synapt G2 mass spectrometer. For chromatographic separation, a 2.1x150 mm Acquity UPLC BEH C18 column, 1.7 µm was operated at 55°C with a constant flow of 0.4 mL/min. The mobile phases consisted of 10 mM aqueous NH_4OAc solutions (A) and acetonitrile (B). Phase B was increased linearly from 5 % (v/v) to 100 % (v/v) over 10 min and held at 100% for 2 min. Mass spectra were recorded at Positive ion mode.

Mass spectrometric analysis of reaction product **11**:



The sample was analyzed with an in-house LC-MS method. The following experimental parameters were used. The LC pump was operated at 0.6 mL/min in gradient mode with a mobile phase A composed of 10mM NH_4Ac per liter. Mobile phase B was acetonitrile. The gradient elution started at 90A/10B and changed linearly over 15 minutes to 100B. The final mobile phase composition was hold for 2 min. The HPLC column was an XBridge C18, 3.5 µm 3.0 x 150 mm type from Waters, maintained at 35ºC in a column oven. Mass spectra were recorded at Negative ion mode.

LC-MS analyses of 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propanoic acid (**10**) were performed on a Waters UPLC system coupled to a Synapt G2 mass spectrometer. For chromatographic separation, a 2.1x150 mm Acquity UPLC BEH C18 column, 1.7 µm was operated at 45°C with a constant flow of 0.4 mL/min. The mobile phases consisted of 10 mM aqueous NH_4OAc solutions (A) and acetonitrile (B). Phase B was increased linearly from 10 % (v/v) to 100 % (v/v) over 10 min and held at 100% for 2 min. Mass spectra were recorded at Positive ion mode.

Scheme 1: Photolytic drug degradation (simplified).

Scheme 2: Formation of TEMPOL by a reaction of 4-OH-TMP with singlet oxygen and its EPR spectrum.

Scheme 3: Degradation of TMC647055 (**1**) upon light irradiation or in a dark singlet oxygen stress test.

Scheme 4: Chromatograms of TMC647055 (**1**) after light irradiation or exposure to dark singlet oxygen.

Scheme 5: Active pharmaceutical ingredients VU0409551/JNJ-46778212-AAA (**4**) and JNJ-54119936-AAA (**5**).

Scheme 6: Light induced degradation of **4** and **5** either in separate vials (a) and (b) or in combination (c).

Scheme 7: **5** serving as a catalyst in the photolytic decomposition process of **4**.

Scheme 8: Time dependent formation of TEMPOL upon irradiation of solutions containing **5** (red) or **4** (blue) in comparison to the background reaction of TEMPOL-formation (black).

Scheme 9: Stability of **5** (a) and **4** (b) in a dark singlet oxygen stress test.

Scheme 10: Preparation of oxidative degradant **11** from antioxidant 10 together with its chromatogram.

Table 1: Formation of TEMPOL upon light irradiation to evaluate photosensitizing potency of **1**.