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Synthesis and Characterization of photoactivatable doxycycline analogues bearing two-photon sensitive photoremovable groups suitable for light induced gene expression

Bastien Goegan,[a] Firat Terzi,[b] Frédéric Bolze,[a] Sidney Cambridge,[*b] and Alexandre Specht [*a]

Abstract: We report herein the synthesis and the photolytic properties of EANBP and PEG7-DEACM caged 9-aminodoxycycline. 9-Aminodoxycycline is a tetracycline analogue able to activate transcription via the inducible TetOn transgene expression system and can be regioselectively coupled by carbamoylation to two-photon sensitive photoremovable protecting groups. The EANBP caged 9-aminodoxycycline showed complex photochemical reactions but did release 10% of 9-aminodoxycycline. However, the PEG7-DEACM-9-aminodoxycycline exhibited excellent photolysis efficiency at 405 nm with a quantitative release of 9-aminodoxycycline and a 0.21 uncaging quantum yield. Due to the good two-photon sensitivity of the DEACM chromophore, 9-aminodoxycyline release is possible by two-photon photolysis with a calculated action cross-sections up to 4.0 GM at 740 nm. Therefore, PEG7-DEACM-9-aminodoxycycline represents a very attractive tool for the development of a light induced gene expression method in living cells.

Introduction

The photochemical regulation of gene function is a rapidly advancing research field in the functional genomics era since the manipulation of complexes tissues, such as interfering with pattern formation during tissue development, requires precise spatial and temporal control of gene expression.[1] Besides fully genetically encoded photoactivated gene expression methods,[2] synthetic photoresponsive molecules are particularly attractive to developmental biologists[3] and neurobiologists.[4]

One of the most commonly used ligand-inducible gene expression systems is the Tet system which takes advantage of an *Escherichia coli* antibiotic resistance mechanism. Because of its nanomolar affinity, the tetracycline-responsive gene regulation system is highly specific and efficient and is thus used widely for transgene expression. Its main element is the homodimeric Tet repressor (TetR).[5] TetR tightly binds its effector tetracycline, or the more potent derivative doxycycline, as a magnesium (II) chelate to a regulatory core domain. This leads to an allosteric conformational change in TetR that results in its dissociation from the operator DNA sequence (TetOff system). Expansion of the biological space for TetR-based expression systems has led to novel eukaryotic transcriptional activators.[6] Random mutagenesis coupled with phenotypic screening has given rise to variants termed rtTA (reverse tetracycline-controlled transactivator), which exhibit a reversed allosteric response and require the tetracycline agonists anhydrotetracycline or doxycycline for DNA binding (TetOn system).[7]

By rendering Tetracycline analogues (and in particular doxycycline) photoactivatable, one has now the potential to develop a method for high spatial and temporal control of gene expression by irradiation with light. To do so, light controlled transcriptional activation has been achieved with photoremovable groups (e.g. caging groups).[1,4] 4,4-Dimethoxy-2-nitrobenzyl-caged doxycycline derivatives have been combined with the TetOn system in order to photoinduce reporter gene expression in several eukaryotic systems including plant leafs, brains slices, as well as living Xenopus tadpoles, and living mouse embryos.[8] However, none of the current photoactivatable doxycycline analogues could be synthesized efficiently and the synthesis protocols were not amenable to large-scale production of the compounds. Previous synthetic strategies involved a coupling reaction without prior protection of the various reactive functions of the doxycycline molecule.[8] Consequently, published synthesis protocols report poor yields and required a substantial effort for purification of the final product. Another drawback of these light-triggered gene expression systems is the use of UV sensitive photoremovable protecting groups (PPG) and consequently low light penetration depth and potential phototoxicity.[9] To overcome the dilemma that only high-energy light can induce photochemical reactions on PPG, two-photon (TP) photolysis was explored to shift from UV-blue to red-IR excitation.[10] Indeed, this nonlinear optical phenomenon allows the use of IR light for excitation leading to a deeper light penetration in biological samples and low phototoxicity. In addition, a higher intrinsic three-dimensional spatial resolution is achieved since the two-photon absorption phenomenon is limited to a very small volume at the focal point of the optical system used (~ 1µm^3). In this context, new generations of PPGs with remarkably high two-photon absorption cross-sections have been recently developed. Here, we present the design, the efficient synthesis, and the

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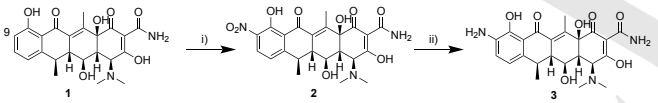
characterization of caged doxycycline analogues using the TP sensitive 2-(4'-((di(tris-ethoxy(methoxy))amino)-4-nitro-[1,1'-biphenyl]-3yl) propan-1-ol (EANBP)[11] as well as a soluble version of the well-known 7-(Dialkylamino)coumarin-4-yl]methyl (DEACM)[12] caging groups.

Results and Discussion

Design and synthesis of 9-aminodoxycycline

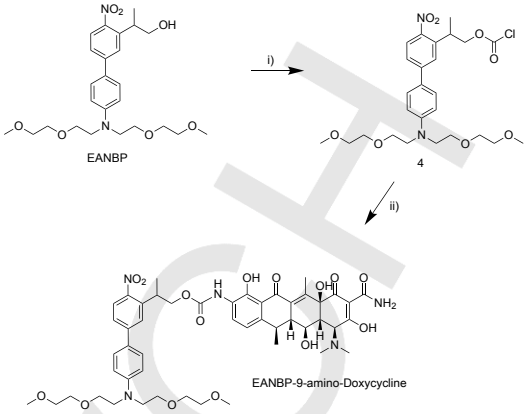
Doxycycline has a complex structure and thus the synthesis of caged doxycycline is difficult. In particular, doxycycline has many nucleophilic functional groups (Scheme 1). To be able to link a PPG to a specific site on doxycycline, we decided to introduce an additional highly nucleophilic group on the doxycycline skeleton. Of course, doxycycline analogues with this new functionality should still be able to induce transgene expression via the TetOn system. For this, we focused on position 9 of the doxycycline ring system. This position was selected based on the X-Ray structure of the doxycycline-Mg^{2+} complex bound to the repressor (TetR).[13] The three-dimensional arrangement of doxycycline-Mg^{2+} complex bound to TetR revealed that position 9 could be further modified, without significantly interfering with the capacity of doxycycline to interact with TetR. Therefore, 9-aminodoxycycline **3** was prepared in two steps starting from commercial doxycycline hyclate **1** (Scheme 1). The later was first nitrated in position 9 using a mixture of concentrated nitric and sulfuric acids leading to 9-nitro-doxycycline **2** with 97 % yield. Subsequently, 9-aminodoxycycline **3** was obtained with 96 % yield after catalytic reduction of **2** using Pd/C in degassed methanol.



Scheme 1: Synthesis of 9-aminodoxycycline : i) H_2SO_4 (conc.), HNO_3, 0°C, 1h, ρ = 97 %, ii) MeOH, Pd/C (cat.), H_2, RT, 1h, ρ = 96 %.

Synthesis of Caged 9-aminodoxycycline derivatives

For an efficient TP uncaging at 800 nm, the EANBP caging group was first selected to produce highly TP-sensitive caged doxycycline analogues. Of note, this later photoremovable group is bearing oligoethylene glycol (OEG) chains in order to improve its water solubility. First, the alcohol of the caging chromophore EANBP was synthesized according to a previously described procedure.[11c] With **3**, the EANBP-9-aminodoxycycline analogue could be synthesized by regioselectively grafting the EANBP-chloroformate **4** to the 9-aminodoxycycline **3** with 50 % yield (scheme 2).

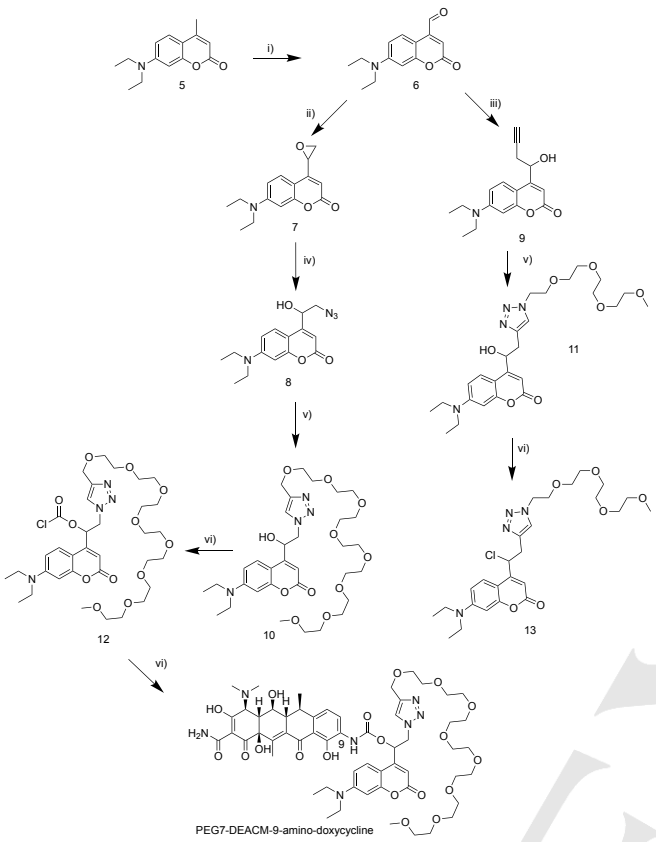


Scheme 2: Synthesis of EANBP-9-aminodoxycycline: i) EANBP, trisphosgene, DIPEA, THF, RT, 1,5 h ii) **3**, $NaHCO_3$, dioxane, H_2O, RT, 2 h, ρ = 50 %.

We decided to also synthesize a second TP sensitive caged-doxycycline analogue, using the well-known DEACM PPG. However, this photoremovable protecting group is very hydrophobic. In order to improve its water solubility, we introduced polyethylene glycol chains (PEG) at the benzylic position of the DEACM caging group using copper catalyzed azide-alkyne cycloaddition (CuAAC) reactions.[14] Therefore, two synthetic pathways have been developed in order to incorporate either a propargyl or an azide function at the benzylic position of the DEACM chromophore (scheme 3). Starting from commercially available 7-(diethylamino)-4-methyl-2*H*-chromen-2-one **5,** the 7-(diethylamino)-2-oxo-2*H*-chromene-4-carbaldehyde **6** was synthesized with 47 % yield using selenium dioxide as an oxidizing agent.[15] The aldehyde **6** was converted to the epoxide **7** with 67 % yield using carboxymethyl-4-cyanophenylmethylsulfonium trifluoromethanesulfonate in the presence of cesium carbonate.[16] The azide containing DEACM analogue **8** was obtained by the opening of the epoxide **7** with 47 % yield using sodium azide. In parallel, the alkyne containing DEACM analogue **9** was obtained with 85 % yield by the reaction of freshly prepared propargyl zincate with the aldehyde **6** as previously reported.[17]

The azide containing DEACM analogue **8** was used in a CuAAC reaction with the mPEG7-propyne (see supporting information for the synthesis) leading to the PEG7-DEACM alcohol **10** with 60 % yield. This later was converted to the corresponding chloroformate **12** in order to regioselectively graft the 9-aminodoxycycline **3** to obtain the PEG7-DEACM-9-aminodoxycycline with 40 % yield. In parallel, the alkyne containing DEACM analogue **9** was coupled to the mOEG4-azide (see supporting information for the synthesis) with 85 % yield. Surprisingly, we were not able to synthesize the chloroformate of **11** since the reaction of **11** with trisphosgene lead to the formation of the chlorinated analogue **13** with 99 %

yield. This indicates that the triazole orientation can influence the reactivity of our soluble versions of the DEACM caging group.



Scheme 3: Synthesis of PEG7-DEACM-9-aminodoxycycline : i) SeO_2, Xylene, reflux, 16 h, ρ = 47 %, ii) $CsCO_3$, (carboxymethyl)(4-cyanophenyl)methylsulfonium, THF, 60°C, 2h, ρ = 67 %, iii) Propargyl bromide, Zn, THF, RT, ρ = 85 %, iv) NaN_3, NH_4Cl, EtOH, H_2O, reflux, 16 h, ρ = 47%, iv) PEG7-propyne or OEG4-azide, $CuSO_4$, ascorbic acid, proline, DMF, *t*-BuOH, H_2O, 60°C, 24 h, ρ = 60-85 %. vi) Trisphosgene, DIPEA, THF, RT, 1,5 h, then $NaHCO_3$, dioxane, H_2O, RT, 2 h, ρ = 40%.

In summary, the 9-aminodoxycycline analogue was used to efficiently and regioselectively graft two photoremovable groups using carbamate linkages to generate EANBP-9-aminodoxycycline and PEG7-DEACM-9-aminodoxycycline, two new caged doxycycline analogues.

Photophysical and Photochemical characterization of caged 9-aminodoxycycline derivatives

The one-photon properties of EANBP-9-aminodoxycycline and PEG7-DEACM-9-aminodoxycycline were investigated by UV-visible spectroscopy. The absorption maxima at pH 7.2 of EANBP-9-aminodoxycycline and PEG7-DEACM-9-aminodoxycycline are 375 nm and 390 nm with molar absorption coefficients of 9 000 $M^{-1}cm^{-1}$ and 13 000 $M^{-1}cm^{-1}$, respectively. Figure 1 shows the evolution of the UV-vis spectrum of EANBP-9-aminodoxycycline (A) and PEG7-DEACM-9-aminodoxycycline (B) during photolysis in phosphate buffer. The isosbestic points at 340 and 450 nm for EANBP-9-aminodoxycycline and at 345 and 445 nm for PEG7-DEACM-9-aminodoxycycline indicate that a clean photochemical reaction occurred leading to stable photoproducts.

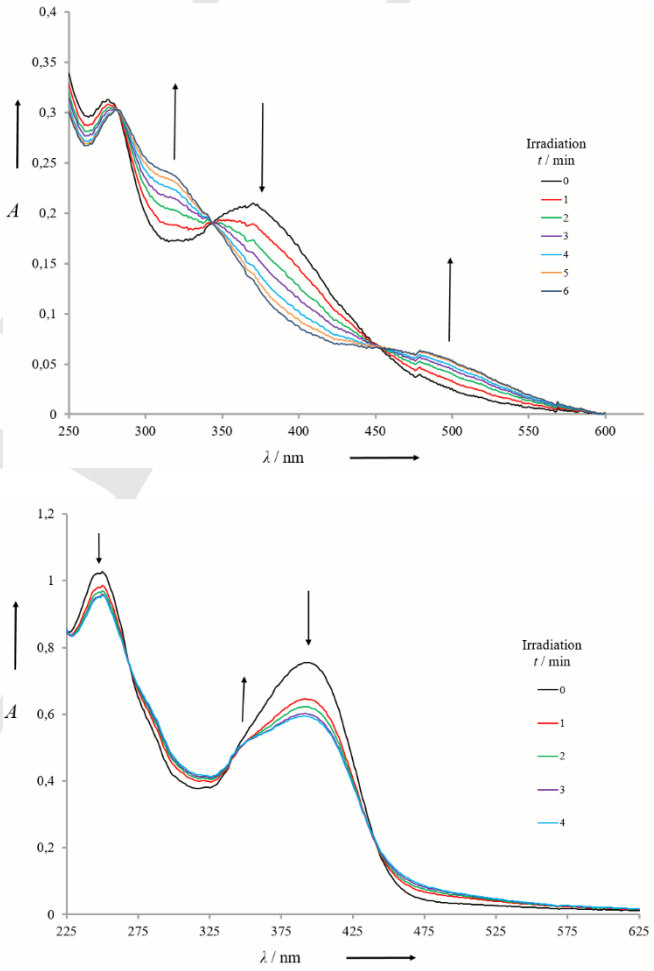
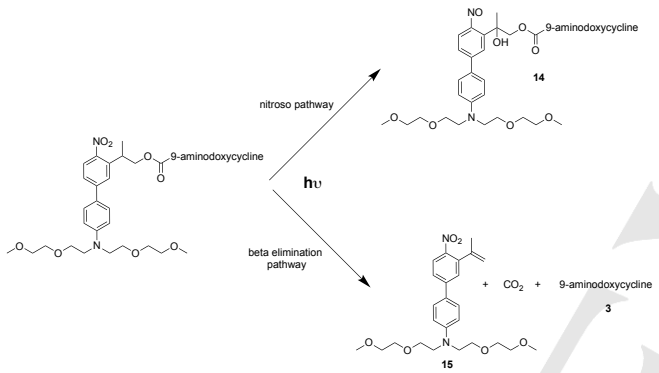


Figure 1 (A) Changes in the UV/visible spectrum of EANBP-9-aminodoxycycline during photolysis (405 nm) in phosphate buffer (25 μM, pH 7.4). (B) Changes in the UV/visible spectrum of PEG7-DEACM-9-aminodoxycycline during photolysis (405 nm) in phosphate buffer (60 μM, pH 7.4).

The photolytic release of 9-aminodoxycycline was analysed quantitatively by HPLC after irradiation in neutral buffered medium. Surprisingly, EANBP-9-aminodoxycycline afforded only a 10 % yield of released 9-aminodoxycycline. Previously, we achieved significantly higher yields with EANBP-

caged GABA[11d] and EANBP-caged Phosphate (Pi).[11c] Thus, these data suggest that uncaging of EANBP-9-aminodoxycycline leads to a product that "hijacks" or blocks 9-aminodoxycycline release with a 90 % probability. This is supported by the observation that the absorption maximum of the EANBP-9-aminodoxycyline shifted from 397 nm to 375 nm compared to EANBP-caged GABA (or Pi). Also, the photochemistry of this class of photoresponsive molecules (i.e. orthonitrophenethyl derivatives) is strongly dependent on solvent and basicity.[18] One could speculate that the doxycycline moiety increased the hydrophobic environment near the EANBP caging group. This could potentially cause a shift of the absorption maximum of the 4-amino-4'-nitro-biphenyl chromophore and, unfortunately, lead to a new major photochemical pathway and a photorearrangement producing a hydroxy-nitroso product **14** (Scheme 4).



Scheme 4. Proposed photochemical pathways for EANBP-9-aminodoxycycline adapted from Woll *et al.* [18b]

The PEG7-DEACM-9-aminodoxycycline in turn exhibited a simple photolysis, since it afforded a quantitative yield of 9-aminodoxycyline**.** A one-photon quantum yield of 0.21 for PEG7-DEACM-9-aminodoxycyline uncaging could therefore be determined by comparison with the DEACM-Gly reference molecule at 405 nm by HPLC analysis.[12c] This later value is in agreement with the good quantum yields observed with ethyl substituted coumarin-4-yl PPG.[19]

Thus, the high photolysis quantum yield and the significant molar absorption coefficient in the blue ($\varepsilon.\Phi$ = 2700 $M^{-1}cm^{-1}$ at 390 nm) together with the very efficient release of 9-aminodoxycycline makes PEG7-DEACM-9-aminodoxycycline a very suitable caged doxycycline derivative for visible light photolysis at neutral pH. Moreover, based on the previously reported two-photon absorption cross-section (δ_a) for the DEACM chromophore of 2,3 GM at 800 nm[20] and the calculated value of 19 GM at 740 nm (see supporting information),[21] the two-photon uncaging action cross-section ($\delta u = \delta_a . \Phi$) of PEG7-DEACM-9-aminodoxycycline is estimated to be 4.0 GM at 740 nm and 0.5 GM at 800 nm. This two-photon uncaging action cross-section of PEG7-DEACM-9-aminodoxycycline is, to our knowledge, not only the highest value reported for a caged doxycycline analogue but the highest for any transcriptionally active small molecule including Tamoxifen and IPTG.

To demonstrate the good two-photon sensitivity of PEG7-DEACM-9-aminodoxycycline, 100 μL of a 100 μM solution in PBS were irradiated for 90 min using a femtosecond laser (Insight Spectra-Physics) at 740 nm. HPLC analysis of the photolyzed sample indicated a 30 % conversion. This underscores the TP-sensitivity of PEG7-DEACM-9-aminodoxycycline and suggests that it is suitable for TP-mediated uncaging *in vivo*.

Transcriptional efficacy of 9-aminodoxycycline and photoactivated 9-aminodoxycycline

To test the transcriptional efficacy of 9-aminodoxycycline, we used dissociated hippocampal neurons which were virally transduced with two components of the TetOn system, i.e. rtTA and a Tet-dependent GFP construct. One week after transduction with adeno-associated viruses (AAV), the doxycycline derivatives were added to the neurons at different concentrations. Compared to unmodified doxycycline, 9-aminodoxcycline had a lower affinity to rtTA as it required roughly five-fold higher concentrations to achieve similar levels of GFP expression (Figure 2 A, B). This indicates that 9-aminodoxycycline is an attractive alternative to doxycycline as it possesses similarly high transcriptional activity while being much more amenable to chemical modification.

Adding PEG7-DEACM-9-aminodoxycycline to Tet system competent neuronal cultures did not produce any appreciable levels of background fluorescence (Figure 2 A, B). In turn, quantitative photoactivation of PEG7-DEACM-9-aminodoxycycline before adding it to the cultures yielded levels of GFP fluorescence that were very similar to those induced by unmodified PEG7-DEACM-9-aminodoxycycline. This strongly suggested that the side product of the photolytic reaction (the coumarin derivative **10**) did not exhibit any significant toxicity as the sensitive gene expression as well as neuronal morphology were not impaired.

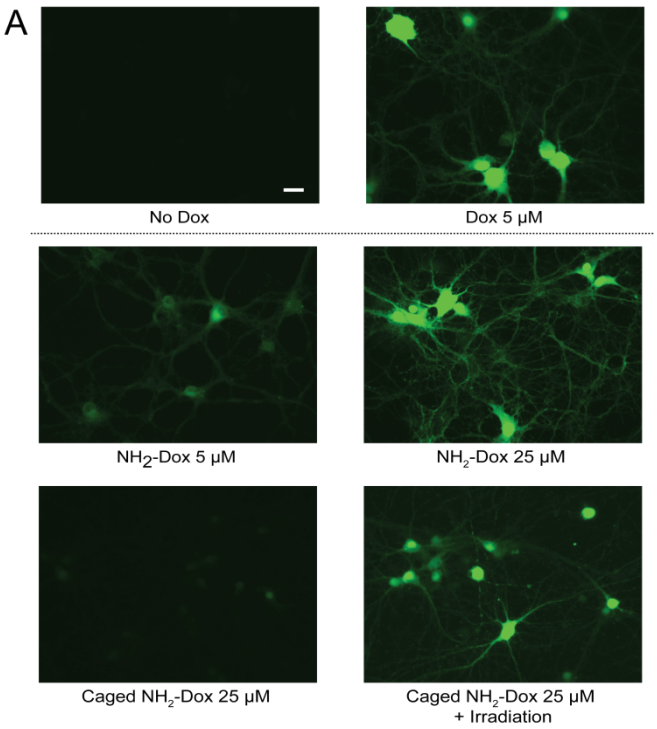


Figure 2. (Photoactivated) transgene induction with doxycycline (Dox) derivatives. (A) Representative images of GFP expression (24 h after induction) with different doxycycline derivatives. One-photon irradiation (430 nm) was performed prior to administration to cells. (Scale bar: 20 μm). (B) Quantification of somatic GFP fluorescence levels in rat hippocampal cultures 24 h after induction. The total fluorescence values within an equal sized region of interest were assessed using NIH ImageJ (n = 20-40 neurons for each condition; error bars: standard deviation; PEG7-DEACM-9-aminodoxycycline = caged NH_2**-Dox)**

Conclusions

There is a sizeable need for two-photon sensitive caged doxycycline derivatives which can be used to induce transgene expression in dense three-dimensional tissue with two-photon irradiation. This lead us to synthesize a new doxycycline analogue, 9-aminodoxycycline, to allow easy and efficient grafting of visible-light and two-photon sensitive photoremovable chromophores onto it (i.e. EANBP and PEG7-DEACM). The 9-aminodoxycyline derivative induces similar transgene expression levels as the 'gold standard' doxycycline, albeit at higher concentrations. 9-aminodoxycyline can be regioselectively coupled by carbamoylation to two-photon sensitive photoremovable groups. While photoactivation of the EANBP-9-aminodoxycycline derivative led to some 9-aminodoxycycline release, most of the desired product is probably consumed by other complex photochemical reactions.

Presumably, a major photochemical pathway leading to a hydroxy-nitroso product "hijacks" 9-aminodoxycycline release. More importantly however, PEG7-DEACM-9-aminodoxycycline showed a very efficient 9-aminodoxycycline release using visible light photolysis ($\varepsilon.\phi$ = 2700 $M^{-1}cm^{-1}$ at 390 nm), or 740 nm two-photon excitation (with a calculated δ_u = 4.0 GM).

In summary, we produced a new caged doxycycline derivative that can be used in combination with the TetOn system for photoactivated gene expression. Our future studies will focus on the use of two-photon microscopy for uncaging of PEG7-DEACM-9-aminodoxycycline in highly scattering tissue such as the brain to induce transgene expression with high spatiotemporal resolution.

Experimental Section

Synthesis

General: All chemicals for caged 9-aminodoxycycline synthesis were analytical grade and purchased from Sigma-Aldrich, Alfa Aesar, or Acros organics. A phenomenex C18 column PolarRP (4.6, 250 mm) was used for HPLC analysis and a phenomenex C18 column PolarRP (10, 250 mm) was used for HPLC purifications. For HPLC analysis or purification, elutions were performed at a flow rate of 1 mL/min or 4 mL/min, respectively, using a linear gradient of acetonitrile in H_20 (0,1 % TFA) from 0 to 100 % (v/v) over 30 min. THF was distilled over sodium under argon; methylene chloride was distilled over calcium hydride under argon. Other anhydrous solvents were purchased from Sigma-Aldrich. All aqueous solutions were prepared with deionized water from a commercial water purifier with a conductivity of 18 MΩ or higher.

^1H and ^{13}C spectra were recorded with a 400 MHz bruker Avance 400 instrument in $CDCl_3$ (internal standard 7.24 ppm for ^1H and 77 ppm, middle of the three peaks, for ^{13}C spectra) or [D_4] MeOD (internal standard 3.31 ppm for ^1H and 49 ppm for ^{13}C spectra). An agilent MM-ESI-ACI-SQ MSD 1200 SL spectrometer or an agilent LC-MS RRLC 1200SL/ESI Qtof 6520 spectrometer was used for ESI analysis. TLCs were run on Merck precoated aluminium plates (Si 60 F254). Column chromatography was performed Merck silica gels (60-120 mesh). Absorption spectra were recorded with a UVIKON XS spectrometer.

EANBP-chloroformate (4): To a stirred solution of EANBP (see supporting information) (63.5 mg, 0,13 mmol) in 1.5 mL of anhydrous THF, triphosgene (39.54 mg, 0,13 mmol) and DIPEA (22.2 µL, 0.13 mmol) were slowly added at 0 °C. The reaction mixture was stirred at room temperature for 90 min. Then the solvent was evaporated to yield the EANBP-chloroformate as a pale yellow oil which was used in the next step without further purification.

EANBP-9-aminodoxycycline: 9-Aminodoxycycline **3** (47.2 mg, 0.10 mmol) and $NaHCO_3$ (21.6 mg, 0.26 mmol) were dissolved in water (5 mL). After the gas development stopped, EANBP-Chloroformate **4** (36 mg, 0.07 mmol) dissolved in dioxane (5 mL) was added at 0°C. The reaction mixture was stirred at room temperature for 2 h. The completion of the reaction was monitored by HPLC analysis. The solvents were removed under vacuum and the residue was purified by RP-HPLC (C18; using a mixture of solvent A: TFA (0.1 %) in H_2O and solvent B: acetonitrile, 91-9 %, FR: 4 mL/min^{-1}, t_R: 10 min) to afford 25 mg (50 % yield) as a yellow powder. Analytical HPLC : t_R : 19.2 min, purity : > 99 % ; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.86 (d, J = 8.5 Hz, 1H), 7.58 (d, J = 2.2 Hz, 1H), 7.46 (m, 3H), 7.15 (d, J = 6.9 Hz), 6.8 (d, J = 6.9 Hz, 1H), 6.78 (d, J = 8.5 Hz, 2H), 3.93 (m, 2H), 3.6 (m, 16H), 3.37 (s, 6H), 3.15 (s, 6H), 1.57 (d, J = 6.9 Hz, 3H), 1.43 (d, J = 7.2 Hz, 3H) ppm.

4-carbaldehyde-7-diethylamino-coumarin (6): Selenium dioxide (960 mg, 8.6 mmol) was added to a solution of 7-Diethylamino-4-methylcoumarin (1 g, 4.3 mmol) in *p*-xylene (42 mL). The resulting mixture was heated at reflux for 16 h until complete consumption of the starting material and was then filtered while hot to remove black selenium. The filtrate was concentrated under reduced pressure and the crude product was purified by column chromatography on silica gel (heptane/ethylacetate (AcOEt) 8/2 in vol.) to yield 4-carbaldehyde-7-diethylamino-coumarin (500 mg, 47 %) as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10 (s, 1H), 8.3 (d, 1H, J = 9.0 Hz), 6.6 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 6.51 (d, 1H, J = 2.4 Hz), 6.43 (s, 1H), 3.4 (q, 4H, J = 7.0 Hz), 1.2 (t, 6H, J = 7.0 Hz) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ = 192.5, 161.8, 157.4, 151.0, 143.9, 126.7, 117.2, 109.7, 103.7, 98.3, 44.8, 12.4 ppm.

7-(diethylamino)-4-(oxiran-2-yl)-2H-chromen-2-one (7): 4-carbaldehyde-7-diethylamino-coumarin **6** (236 mg, 0.96 mmol) and cesium carbonate (470 mg, 1.44 mmol) were dissolved in THF (4 mL) and heated at 60 °C for 10 min. Then a suspension of (carboxymethyl)(4-cyanophenyl)methylsulfonium (300 mg, 1.44 mmol) (as prepared by Forbes *et al.* [16]) in THF (6 mL) was slowly added to the reaction mixture and the reaction was heated at 60°C for 2 h. The solution was cooled at room temperature, filtered through celite, concentrated under reduced pressure, dried over Mg_2SO_4, and purified by column chromatography on silica gel (heptane/AcOEt: 7/3 in vol.) to give the targeted compound with 67 % yield (173 mg) as an orange-red oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.5 (d, 1H, J = 9.0 Hz), 6.6 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 6.51 (d, 1H, J = 2.4 Hz), 6.02 (s, 1H), 4.04 (m, 1H), 3.4 (q, 4H, J = 7.0 Hz), 3.21 (m, 1H), 2.7 (m, 2H), 1.2 (t, 6H, J = 7.0 Hz) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ = 162, 156.2, 151.9, 150.8, 124.6, 108.7, 106.9, 103.9, 97.8, 49.9, 48.6, 44.8, 12.5 ppm; MS (ESI): calculated for $C_{15}H_{17}NO_3$: 259.121, found 259.013.

4-(2-azido-1-hydroxyethyl)-7-(diethylamino)-2H-chromen-2-one (8): To a mixture of **7** (173.2 mg, 0.67 mmol) in Ethanol (2 mL) was added NaN_3 (52.11 mg, 0.8 mmol), NH_4Cl (36.8 mg, 0.69 mmol), and water (2 mL). The mixture was stirred at reflux for 16 h, then the crude product was filtrated to remove NaN_3 excess and EtOH was removed by rotary evaporation. H_2O (10 mL) was added and the solution was extracted with Diethyl Ether (Et_2O) 3×15 mL). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The resulting oil was purified by column chromatography on silica gel (Heptane/AcOEt : 3/7 in vol.) to produce the targeted compound with 47 % yield (125.5 mg) as a clear red oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.3 (d, 1H, J = 9.0 Hz), 6.5 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 6.48 (d, 1H, J = 2.4 Hz), 6.3 (s, 1H), 5.1 (m, 1H), 3.5 (m, 2H), 3.4 (q, 4H, J = 7.0 Hz), 2.7 (d, 1H, J = 3.6 Hz), 1.2 (t, 6H, J = 7.0 Hz) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ = 162.6, 156.7, 154.4, 150.7, 148.3, 124.5, 108.9, 106.3, 105.8, 98.3, 69.6, 56.6, 45.1, 12.5 ppm; MS (ESI): calculated for $C_{15}H_{18}N_4O_3$: 302.138, found 302.042.

PEG$_7$-DEACM (10): mPEG$_7$-propyne (see supporting information) (150 mg, 0.40 mmol), compound **8** (126.1 mg, 0.42 mmol), ascorbic acid (34.9 mg, 0.19 mmol), and proline (9.1 mg, 0.08 mmol) were dissolved in a mixture of DMF/*t*-butanol/water (1:15:1 in vol.). The mixture was degassed by three freeze–pump–thaw cycles, then copper sulfate (12.7 mg, 0.08 mmol) was added. The reaction mixture was stirred at 60 °C and monitored by TLC/HPLC until all starting material disappeared (approximatively 24h). After completion of the reaction, the mixture was diluted with water and extracted with dichloromethane (3x). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The reaction products were purified using silica gel column chromatography (CH_2Cl_2/CH_3OH: 1/0 to 96/4 in vol.) and revealed with phosphomolybdic acid (PMA) TLC stain to give the targeted compound with 69 % yield (185 mg) as an orange oil. Analytical HPLC : t_R : 16.2 min ; UV : 390 nm ; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.88 (s, 1H), 7.53 (d, 1H, J = 8.8 Hz), 6.61 (dd, 1H, J = 8.8 Hz, 2.4 Hz), 6.5 (s, 1H), 6.34 (s, 1H), 5.35 (d, 1H, J = 9.1 Hz), 4.8 (d, 1H, J = 13.5 Hz), 4.69 (s, 2H), 4.29 (m, 1H), 3.3-3.7 (m, 32H), 3.32 (s, 3H), 1.19 (t, 6H, J = 7.0 Hz) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ = 162.7, 156.6, 154.8, 150.9, 145, 124.7, 109.1, 106.2, 105.9, 97.9, 72.1, 70.6, 69.8, 68.9, 64.6, 59.2, 56.1, 44.9, 12.6 ppm; MS (ESI): calculated for $C_{33}H_{52}N_4O_{11}$: 680.363, found 680.275.

PEG$_7$-DEACM-chloroformiate (12): To a stirred solution of PEG$_7$-DEACM **10** (56 mg, 0.08 mmol) in 1.5 mL of anhydrous THF, triphosgene (26.9 mg, 0.09 mmol) and DIPEA (15 µL, 0.06 mmol) were slowly added at 0 °C. The reaction mixture was stirred at room temperature for 90 min, then the solvent was removed by rotary evaporation to yield the targeted compound as a pale yellow oil. It was used for the next step without further purification to minimize hydrolytic processes.

PEG$_7$-DEACM-9-aminodoxycycline: 9-Aminodoxycycline 3 (34.8 mg, 0.07 mmol) and $NaHCO_3$ (15.9 mg, 0.07 mmol) were dissolved in water (1 mL). After gas development stopped, PEG$_7$-DEACM-chloroformiate **11** (56.2 mg, 0.07 mmol) dissolved in dioxane (2 mL) was added at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The completion of the reaction was monitored by HPLC analysis. The solvents were removed under vacuum and the residue was purified by RP-HPLC (C18 using a mixture of solvent A: TFA (0.1 %) in H_2O and solvent B: acetonitrile, 91-9 %, FR: 4 mL/min^{-1}, t_R: 9 min) to afford 35 mg (40 % yield) as a yellow powder. Analytical HPLC : tR : 17.3 min ; UV : 345, 390 nm ; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.8 (s, 1H), 7.49 (d, 1H, J = 8.5 Hz), 6.72 (d, 1H, J = 8.2 Hz), 6.59 (dd, 1H, J = 8.5 Hz, 2.5 Hz), 6.4 (s, 1H), 6.25 (d, 1H, J = 8.5 Hz),, 5.97 (s, 1H), 4.79 (d, 1H, J = 13.5 Hz), 4.64 (m, 1H), 4.54 (s, 2H), 3.44-3.56 (m, 28H), 3.32 (q, 4H, J = 7.0 Hz), 2.85 (s, 6H), 1.12 (t, 6H, J = 7.0 Hz) ppm; MS (ESI): calculated for $C_{56}H_{75}N_7O_{20}$: 1165.506, found 1165.328.

One photon photolysis

A solution of EANBP-9-aminodoxycycline or PEG7-DEACM-9-aminodoxycycline (respectively at 25 and 60 µM) in 10 mM phosphate buffer (4 mL), pH 7.4 was exposed to a LUMOS 43 LED source (Atlas Photonics Inc.) at 405 nm (Typical optical output: 200 mW/cm^2). The reaction was monitored by UV and aliquots of samples (100 µL) were

analyzed by HPLC to determine the percentage of released 9-aminodoxycycline using a calibration curve (see supporting information). The quantum yield for the photoconversion was determined in phosphate buffer (10 mM, pH 7.4) at 25°C by comparison with the photolysis of DEACM-Gly (Φ = 0.11)[12c] of the same concentration (50 μM) as reference. Light (405 ± 0.2 nm) from a 1000 W Hg lamp from Hanovia was focused on the entrance slit of a monochromator for photolysis. Aliquots (100 μL) were subjected to reversed-phase HPLC to determine the extent of the photolytic conversions. Quantum yields were calculated by considering conversions up to 20 %, to limit as much as possible errors due to undesired light absorption during photolysis.

Two-photon photolysis

A 100 μM solution of EANBP-9-aminodoxycycline in 10mM phosphate buffer (100 μL), pH 7.4 was irradiated for 90 min with a femtosecond laser (Insight Spectra-Physic). The beam was focused at the center of a microcuvette (Helma 105-201). The average power of the laser was set to 150 mW. Irradiated (90 min) and non-irradiated samples (40 μL)were analyzed by HPLC (n=2).

Tet-dependent GFP expression in virally-transduced hippocampal cultures

Neuronal cultures of rat hippocampus were prepared by standard procedures according to state regulations.[22] Briefly, hippocampi were isolated at embryonic day 18.5, mechanically and enzymatically (trypsin) dissociated, and plated on poly-lysine coated glass coverslips. We typically plate 30.000 cells per well in 24-well plates. Neurons were cultured in Neurobasal medium with 1x B27, 0.5 mM L-Glutamine, and 1x penicillin/streptomycin (all reagents purchased from Thermo Fisher). Cultures were maintained at 37°C, 5 % CO_2 for one week and were then transduced with AAVs at concentrations sufficient to achieve robust Tet-dependent GFP expression. Per well, about 2.2 x 10^8 of infectious units providing rtTA and 8.9 x 10^8 units with the Tet-dependent GFP AAV were added to the cultures. One week later, the different doxycycline analogues were administered to the neurons. Photoactivation of PEG7-DEACM-9-aminodoxycycline was performed at 430 nm for 30 minutes using a LUMOS 43 LED source (Atlas Photonics Inc.) in 0.5 mL volume of 0.25 mM concentration in Dulbelcco's buffer without $MgCl_2$ and $CaCl_2$. GFP expression was quantified by manually placing an equal size region of interest around the neuronal somata and assessing the fluorescence using ImageJ (NIH).

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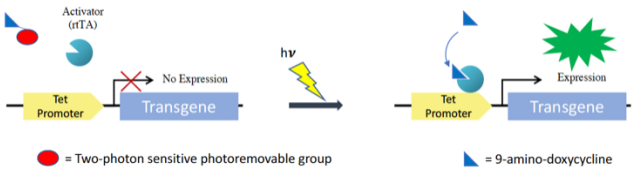

The development of photolabile, i.e. caged versions of 9-aminodoxycycline, which photolyse after visible light irradiation or 740 nm two-photon excitation, are reported herein. The 9-aminodoxycycline is a tetracycline analogue that can be efficiently coupled by carbamoylation to photoremovable protecting groups, leading to new caged doxycycline derivatives that can be used in combination with the TetOn system for photoactivated gene expression.

Bastien Goegan, Firat Terzi, Frédéric Bolze, Sidney Cambridge, and Alexandre Specht**

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Synthesis and Characterization of photoactivatable doxycycline analogues bearing two-photon sensitive photoremovable groups suitable for light induced gene expression