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A NADPH substitute for selective photo-initiation of reductive bioprocesses *via* two-photon induced electron transfer†

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A NADPH substitute where the nicotinamide moiety is replaced by a chromophoric unit having much larger two-photon absorption cross-section and able to transfer electrons to flavins only upon excitation is described as an effective two-photon nanotrigger for selective photo-activation of electron transfer in bioreductive processes.

NADPH is an important protein cofactor that plays an ubiquitous role in numerous bioprocesses. NADPH acts as the electron donor in a number of reductive biosyntheses such as fatty acid and nucleic acid synthesis. It is involved in the electron transfer chain of several proteins including cytochrome P450 reductase (CPR),[1] NO-synthase (NOS),[1–3] ferredoxin reductase where it acts as a primary electron donor. The electron transfer directed mostly towards flavin units (such as FAD in the case of flavocytochrome P450 BM3[4] or NO-synthase) is thus of fundamental importance as a crucial step in the enzymatic machinery.

A molecular tool that would mimic NADPH in terms of protein affinity (*i.e.* binding to NADPH site) but would deliver electron transfer to adjacent electron acceptors such as flavins only upon appropriate activation would be of particular interest. Such a compound could be used to trigger the primary electron transfer in NADPH proteins *in situ* on demand. As such, it could be used as a "nanotrigger" that would allow molecular synchronization of an ensemble of proteins in solution providing an interesting alternative to single molecule addressing. Using laser pulses as the external command is particularly appealing for such a goal. This could be put into practice by designing a nanotrigger that undergoes photo-induced electron transfer to flavins upon photo-excitation. The selectivity and efficiency of photo-excitation are of course key parameters. With this aim in mind, our strategy has been to design a nanotrigger that allows efficient and selective photo-activation *via* two-photon excitation. Indeed two-photon excitation would provide a number of advantages for *in situ* excitation[5] which include (i) intrinsic 3D resolution, (ii) improved penetration depth in biological media (by using NIR excitation sources in the 700–1200 nm range thus reducing scattering and minimizing absorption) and (ii) higher selectivity and lower photo-damage (by decreasing significantly the excitation of endogenous chromophores such as tyrosine, tryptophane, flavins…).

The design of such molecular tool is detailed in Fig. 1. The NADPH substitute is based on the combination of (i) a chromophoric unit that becomes strongly reductive upon excitation and has a significant two-photon absorption (TPA) cross-section in the red–NIR region (that allows better penetration for *in situ* experiments, typically in the 700–1000 nm range) and (ii) a molecular recognition unit that allows selective and efficient recognition of the NADPH binding site. The overall shape and length of NADPH is preserved. An adenosine unit is used as the recognition moiety and the chromophoric moiety is a bis-donor diphenyl-butadiene. A structurally-related symmetrical lipophilic chromophore[6] bearing two dialkylamino end-groups (*i.e.* NBu_2) was previously reported to have a suitable redox potential (*i.e.* 0.44 V/SCE in the ground state for the $\mathbf{C}^{2+}/\mathbf{C}$ couple and −1.04 V/SCE in the excited state for the $\mathbf{C}^{2+}/\mathbf{C}^*$ couple) thus allowing photo-induced electron transfer to a FAD moiety (E° = −0.06 V/NHE). Such a chromophore is also expected to have significantly larger TPA cross-section in the red–NIR region,[7] than NADPH[8,9] and adenine[10,11] thus allowing efficient two-photon excitation and triggering of electron transfer. The replacement of one of the dialkyamino group by a NH_2 unit although expected to lead to a slight reduction in maximum TPA cross section as compared to its lipophilic symmetrical analogue, was meant to enhance the water-solubility and reinforce the affinity for the NADPH binding site in proteins.

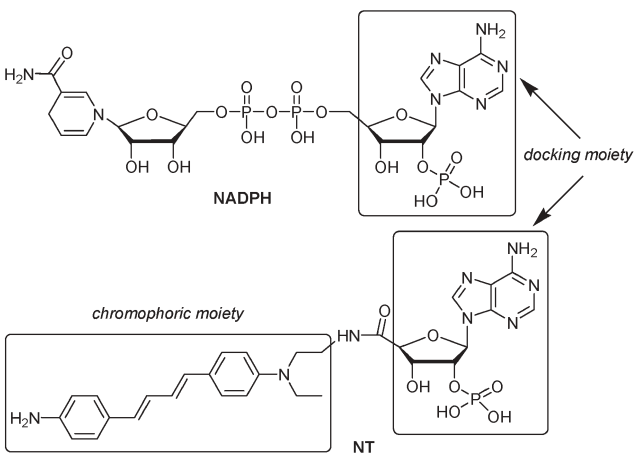

Fig. 1 Design of a NADPH substitute for selective two-photon triggering of bioreduction.

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† Electronic supplementary information (ESI) available: Synthesis of nanotrigger **NT** and experimental details of spectroscopic measurements. See DOI: 10.1039/b615628b

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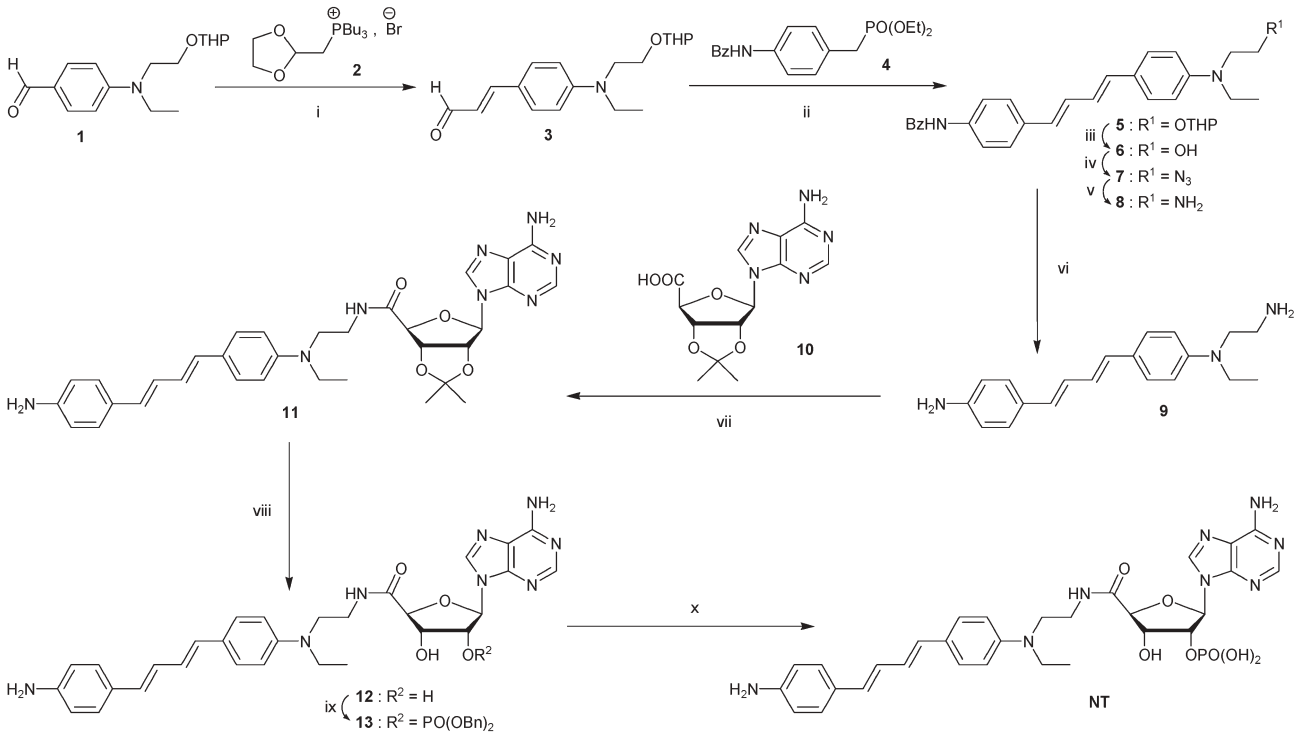

Scheme 1 i: a, **2**, THF, NaH; b, AcOH, CH_2Cl_2 (92%); ii: **4**, NaH, THF (63%); iii: HCl, CH_2Cl_2 (95%); iv: a, TsCl, pyridine; b, NaN_3, *N*,*N*-dimethylacetamide (69%); v: a, Ph_3P, toluene; b, THF, H_2O (100%); vi: NaOH, EtOH (75%); vii: **10**, HBTU, DMF, Et_3N (57%); viii: TFA, H_2O, THF (96%); ix: a, $(iPr)_2N$–$P(OBn)_2$, tetrazole; b, O_2 (69%); x, Me_3SiBr, CH_2Cl_2 (88%).

The synthesis of the NADPH substitute **NT** is described in Scheme 1. The chromophoric moiety **9** was prepared in a six-step sequence from aldehyde **1**,[12] which was first reacted with tributyl[(1,3-dioxolan-2-yl)methyl]phosphonium bromide[13] (**2**), to afford, after hydrolysis under mild conditions, the homologous α,β-unsaturated aldehyde **3**. Horner-Emmons-Wadsworth condensation of **3** with phosphonate **4**[14] gave stereospecifically the *E*,*E*-diene **5**. The tetrahydropyranyl protective group was then removed under acidic conditions, and the resulting alcohol (**6**) was then converted into amine (**8**), by reduction of the intermediate azide **7**. The chromophoric moiety **9** was obtained after cleavage of the benzamide group under basic conditions. The coupling reaction between **9** and the adenosine moiety **10**[15] in the presence of HBTU afforded **11**, the isopropylidene group of which was removed with trifluoroacetic acid. The phosphate group was then introduced on **12** with dibenzyldiisopropylphosphoramidite. The resulting phosphoramidate was oxidized, and the benzyl protective groups were finally removed using bromotrimethylsilane to afford **NT** (Scheme 1).

NT is water-soluble and displays intense absorption in the near UV region (Fig. 2). Its absorption maximum is slightly hypochromically and hypsochromically shifted with respect to the symmetrical lipophilic analogue of its chromophoric part (whose absorption peaks at 398 nm in CH_2Cl_2),[6] in agreement with the lower electron-donating ability of the NH_2 group as compared to NBu_2. Yet, due to the presence of this chromophoric unit, the absorptivity of **NT** in the near UV is about *one order* of magnitude larger than that of NADPH (Table 1).

NT emits slight fluorescence (Table 1) which shifts from the visible blue in organic solvents to visible green in water (Fig. 2). Moreover, when excited in the NIR range (700–1000 nm), **NT** generates fluorescence, demonstrating that **NT** can be effectively photo-excited *via* a TPA process.

Two-photon excited fluorescence (TPEF) experiments solution were then conducted to determine the TPA spectra of **NT**. The experiments demonstrate that **NT** exhibits much larger TPA cross-sections than both NADPH and flavins (Fig. 3) as well as other

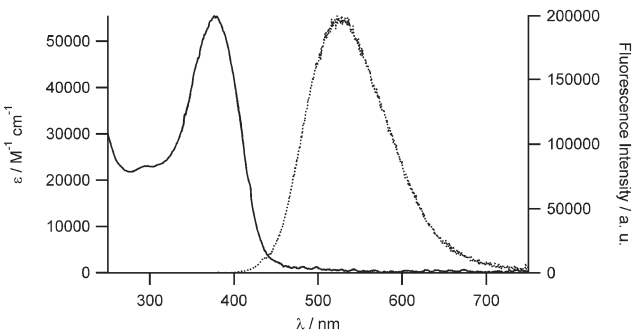

Fig. 2 Absorption and emission spectra of nanotrigger **NT** in H_2O.

Table 1 Photophysical characteristics of nanotrigger **NT** compared to that of **NADPH**

	λ_{abs}/nm	ε/M^{-1} cm^{-1}	λ_{em}/nm	Φ^a
NT	376	60 000	517	0.14
NADPH	342	6 600	450	0.019[16]

[a] Fluorescence quantum yield determined relative to fluorescein in 0.1 N NaOH.

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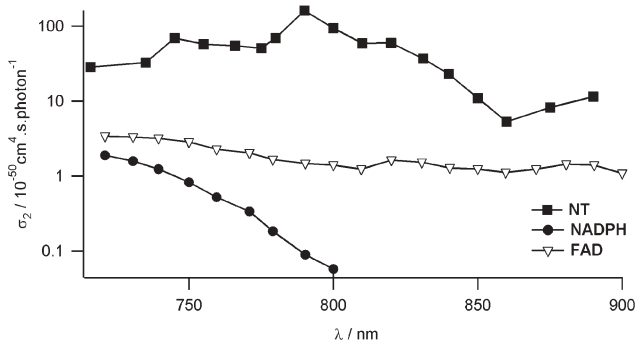

Fig. 3 Two-photon absorption spectra of nanotrigger **NT**, NADPH[8] and FAD[8] in Tris buffer (pH 7.6).

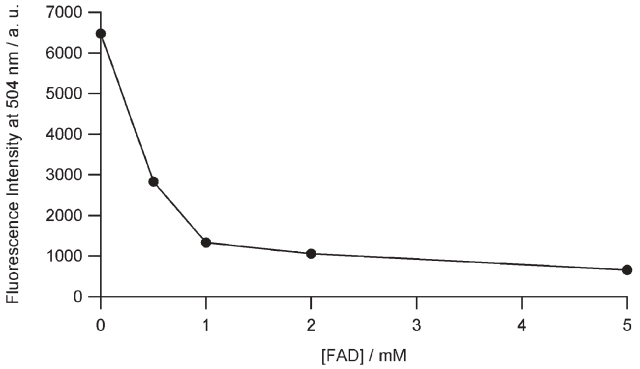

Fig. 4 Quenching of the TPEF fluorescence (excited at 790 nm) of **NT** (1 mM in Tris buffer) as a function of the concentration of FAD.

endogenous chromophores (including amino-acids).[17] This validates the molecular design in terms of effective and selective excitation of the NADPH substitute *via* two-photon excitation in biological samples. At 790 nm, **NT** has a 1800 times larger TPA cross-section than NADPH and 110 times larger than FAD. Interestingly, we observe that **NT** shows a larger TPA cross-section—in the 750–800 nm region (which corresponds to the one-photon allowed transition)—than its symmetrical lipophilic analogue.[7] Hence although the NH_2 group is a less effective donating group than NR_2, the breaking of centro-symmetry induced by the replacement of one NR_2 end-group by a NH_2 group has a positive effect on the magnitude of TPA in the target spectral window for biological applications because the one-photon transition (corresponding to the lowest energy excited-state) is no longer two-photon forbidden.

Cyclic voltammetry experiments conducted in acetonitrile/0.1 M Bu_4NPF_6 show that the chromophoric moiety of **NT** (*i.e.* chromophore **9**) undergoes a two-electron oxidation at 0.61 V/SCE. The oxidation potential is shifted to a more positive value as compared to its symmetrical lipophilic analogue. This is a consequence of the lower electron-donating ability of the NH_2 end-group as compared to the NBu_2 moiety. Chemical oxidation of chromophore **9** in solution‡ shows that chomophore **9** undergoes oxidation to a dicationic species strongly absorbing in the visible region (λ_{max} = 573 nm) which is close to that of its symmetrical lipophilic analogue (λ_{max} = 583 nm).[6] The redox potential of the $\mathbf{9}^{2+}/\mathbf{9}^*$ couple (≈ -0.6 V/NHE estimated from the oxidation potential and absorption and emission properties) suggests that photo-induced electron transfer from **NT** to adjacent FAD is thermodynamically favourable.

Finally, the ability of **NT** to transfer electrons to a flavin unit upon two-photon excitation was tested by exciting at 790 nm **NT** in a Tris buffer solution in the presence of FAD. As shown in Fig. 4, a decrease of the two-photon induced fluorescence signal is observed upon addition of aliquots of FAD as a result of the quenching of the fluorescence of **NT** by FAD. Similar experiments conducted with riboflavin give the same results. This is in agreement with a fast electron-transfer occurring from excited **NT** to FAD. Actually transient absorption experiments conducted on **NT** bound to an endothelial Nitric Oxide Synthase protein (eNOS) confirmed that a fast electron transfer to an adjacent FAD occurs upon one-photon excitation of **NT**.[18] The present study indicates that such process could be triggered selectively using two-photon excitation.

In conclusion we have designed and synthesized a NADPH substitute that allows efficient two-photon excitation induced electron transfer to flavins As such it represents a unique tool for triggering the primary electron transfer in a variety of proteins involved in bioreductive processes with low photo-damage and excellent spatial resolution.

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Notes and references

‡ Performed following the same protocol as described in the literature.[6]

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