Supporting information for

Photocaged permeability: a new strategy for controlled drug release

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1. General

All reagents were purchased from Sigma-Aldrich, Fisher Scientific or TCI America unless otherwise specified and were used as received. Dimethyl Formamide (DMF) used as a solvent for chemical synthesis was dried by vacuum distillation. All reactions were carried out in oven dried glassware and under Ar or N₂ atmosphere. The reactions were carried out in foil-wrapped flasks, protected from light. Flash chromatography was performed using Flash Silica Gel (32-63µ). \(^{1}H\) NMR/\(^{13}C\) spectra were recorded on 400 MHz Bruker AVANCE and 300 MHz Varian instruments. HPLC purification was carried out using a Shimadzu Prominence system using Vydac (218TP C18 5µ) column using 0.1% TFA in acetonitrile and water as eluents and was monitored at \(\lambda_{\text{max}} = 480\) nm. M/S data was collected using Micromass MALDI/TOF (positive and negative modes). The UV lamp used in all studies consisted of a simple aquarium light fixture containing two Philips PL-S 9w/2P BLB bulbs.

The Esophageal cancer cell line (JH-EsoAd1) was cultured at 37°C and in a humidified atmosphere of 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For 96 well plate experiments, the JH-EsoAd1 cells were seeded at 4450 cells/well in 100µL of media in 96 well plates; experiments were carried out one day after seeding. MTT assays were analyzed using a Bio-Tex μQuant plate reader at 562 nm. FACS was performed BD FACS Aria II using BD FACS Diva software at the VCU Flow Cytometry Core Facility. A minimum of 20,000 cells within the gated region were analyzed. Confocal microscopy images were acquired at room temperature with a Leica confocal laser scanning microscope.
List of Abbreviations:

NHS    N-Hydroxysuccinimide
EDC. HCl  (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)
EDANS. HCl  (5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid) hydrochloric acid
Dox    Doxorubicin Hydrochloride
TBTA   tris-(Benzyltriazolylmethyl)amine
TFA    Trifluoroacetic acid
MTT    (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
2. Detailed scheme for the synthesis of EDANS-Dox

\[ \text{NHS, EDC, HCl, DMF} \quad \text{b) propargylamine, N(Et)\textsubscript{3}, DMF} \quad \text{c) Bis 4-nitrophenol carbonate, N(Et)\textsubscript{3}, DMF} \quad \text{d) doxorubicin, HCl, N(Et)\textsubscript{3}, DMF} \quad \text{e) NHS, EDC, HCl, DMF} \quad \text{f) EDANS, HCl, N(ipr)\textsubscript{2}Et, DMF} \quad \text{g) CuSO\textsubscript{4}. 5H\textsubscript{2}O, sodium ascorbate, TBTA, (1:1) DMSO: Water} \]
3. Experimental Methods

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate (1a)

4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy) butanoic acid (1) (200 mg, 0.67 mmol) was dissolved in 10 ml of DMF and stirred for 5 minutes at 0 °C. To this mixture, EDC-HCl (1.5 eq., 156 mg, 1.0 mmol) was added followed by NHS (1.5 eq., 115 mg, 1.0 mmol). The reaction was stirred under N₂ atmosphere in the dark at 0°C for approximately 1 h and then at rt for 15 h. DMF was removed in vacuo. To this mixture, 30 ml of EtOAc was added followed by extraction with water (3x 20 ml). The organic layer was dried with MgSO₄, filtered and the solvent was removed under reduced pressure. The product obtained was single spot on TLC and was taken to the next step without purification.

Synthesis of 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)-N-(prop-2-yn-1-yl)butanamide (2):

1a (250 mg, 0.63 mmol) was dissolved in 5 ml of DMF and stirred for 5 minutes. To this solution triethylamine (2 eq., 175 µL, 1.26 mmol) was added dropwise followed by propargylamine (2 eq., 70 mg, 1.26 mmol). The reaction was stirred overnight at rt, under nitrogen gas in the dark. Then, DMF was removed under low vacuum. To the resulting pale yellow oil was added EtOAc (25 ml), followed by washing with water (3 x 20 mL). The organic
layer was dried over MgSO₄ and filtered; the solvent was removed and purified using column chromatography (100% EtOAc) to yield pale white solid (153 mg, 72%).

**¹H NMR (CDCl₃, 400 MHz):**

δ = 7.57 (s, 1H, Ar-H), 7.31 (s, 1H, Ar-H), 5.93 (br. s, 1H, N-H), 5.55-5.58 (m, 1H, CH₃-CH), 4.12 (t, 2H, -CH₂, J = 5.6 Hz), 4.06 (dd, 2H, -CH₂, Jₐ₋ₜ = 2.4 Hz, Jₜ₋ₜ = 5.2 Hz ), 3.9 (s, 3H, -OCH₃), 2.45 (t, 2H, -CH₂-, J = 6.8 Hz), 2.21 (br.s, 1H, -CH), 2.2 (m, 3H), 1.56 (d, 3H, -CH₃, J = 4.4 Hz).

**¹³C NMR (CDCl₃, 75 MHz):**

δ = 24.7, 24.9, 29.3, 32.7, 56.5, 65.7, 68.6, 71.7, 79.8, 108.9, 109.2, 137.9, 139.4, 146.8, 154.14, 172.5.

MS (MALDI-TOF+): C₁₆H₂₀N₂O₆Na; calculated. (M+Na⁺) 359.12, found= 359.27

Synthesis of 1-(5-methoxy-2-nitro-4-(4-oxo-4-(prop-2-yn-1-ylamino)butoxy)phenyl)ethyl (4-nitrophenyl) carbonate (2a):

To a stirred solution of 2 (100 mg, 0.30 mmol) in dry DMF (10 ml) was added triethylamine (83 μL, 2 eq., 0.59 mmol) followed by bis (4-nitrophenyl)carbonate (271 mg, 3 eq., 0.89 mmol). The solution was stirred overnight in the dark at rt after which it was cooled and acidified with 20 mL of 1% HCl. The mixture was extracted with 30 mL of EtOAc and the organic layer was washed with saturated NaHCO₃ (3x20 mL) solution and brine (1x 20 mL) and dried over MgSO₄. The
solution was filtered, concentrated and purified by flash column chromatography (1:1 to 7:3 EtOAc: hexanes) to yield pale yellow colored solid (81 mg, 54%).

**1H NMR (CDCl₃, 400 MHz):**

δ = 8.26 (d, 2H, Ar-H, J = 9.2 Hz), 7.61 (s, 1H, Ar-H), 7.36 (m, 2H, Ar-H), 7.12 (s, 1H, Ar-H), 6.53 (q, 1H, CH₃-CH, J = 6.4 Hz), 5.91 (br. s, 1H, N-H), 4.14 (t, 2H, -CH₂, J = 6.0 Hz), 4.06 (dd, 2H, -CH₂, J₁₂ = 2.4 Hz, J₁₃ = 5.2 Hz), 4.01 (s, 3H, -OCH₃), 2.45 (t, 2H, -CH₂, J = 7.2 Hz), 2.21 (m, 3H), 1.78 (d, 3H, -CH₃, J = 8.0 Hz).

**13C NMR (CDCl₃, 100 MHz):**

δ = 21.9, 24.6, 29.2, 32.5, 56.54, 68.49, 71.5, 73.7, 79.5, 108.0, 109.1, 115.7, 121.66, 131.4, 139.9, 145.4, 147.6, 151.4, 154.1, 155.3, 171.8.

MS (MALDI-TOF+): C₂₃H₂₃N₃O₁₀Na; calculated (M+Na)+ = 524.13, found = 524.29

![Molecule structure](image)

**Synthesis of photocaged doxorubicin (3):**

To a stirred solution of 2a (80 mg, 0.16 mmol) in dry DMF (3 ml) was added triethylamine (45 μL, 2 eq., 0.32 mmol) followed by Dox (44 mg, 0.5 eq., 0.08 mmol). The solution was stirred at rt for 24 h under nitrogen, in the dark. Then, DMF was removed under low vacuum. To this red colored solid, 30 ml of EtOAc was added and washed with water (3 x 20 ml). The organic layer was dried over MgSO₄, filtered and the solvent was removed and purified using column chromatography (silica/EtOAc and then 1:10 MeOH: CHCl₃) to yield 52% of a mixture of
diastereomers. The resulting solution was purified on RP-HPLC using isocratic elution of 20 %
organic eluent ( 0.1 % TFA in acetonitrile) for 5 minutes and then using gradient elution from
20% to 70% for 30 minutes. The two diastereomers eluted at 35.8 and 36.9 min (See ESI figure
S1). The NMR analysis showed the presence of two products corresponding to the two
diastereomers.

\textsuperscript{1}H NMR (DMSO-d\textsubscript{6}, 400 MHz): (2x means both diastereomeric products had
indistinguishable ppm values for those protons)

14.05 (s,1H), 14.0 (s,1H), 13.27 (s, 2x1H), 7.92 (m, 2x2H), 7.66 (m, 1x2H), 7.54 (s, 1H), 7.49
(s,1H), 7.17 (s, 1H), 7.13 (s,1H), 6.05 (m, 2x1H), 5.41 (m, 2x1 H), 5.21-5.23 (m, 2x1H), 4.95
(m, 2x1 H), 4.78-4.84 (m,2H), 4.66 (m, 2x1), 4.55 ( m, 2x2H), 3.82-4.14 (m, 2x11H), 3.64-3.73
(m, 4H), 3.51 (m,1x2 H), 3.05 (t, 1H, \textit{J} = 2.4 Hz), 3.08 (t, 1H, \textit{J} = 2.4 Hz), 2.97 (m, 4H), 2.90 (s,
1H), 2.74 (s, 1H) 2.18-2.28 (m, 6H), 1.83-1.85 (m, 4x2H), 1.48 (m, 3x2H), 1.10 (m, 3x2H).

\textsuperscript{13}C NMR (DMSO, 100 MHz):

21.92, 27.78, 46.91, 46.99, 56.15, 56.23, 56.26, 56.42, 60.18, 63.7, 66.73, 68.21, 69.74, 72.73,
74.89, 81.21, 100.2, 108.44, 108.66, 110.41, 110.56, 118.8, 118.83, 119.47, 119.73, 128.6,
133.54, 134.4, 135.2, 135.27, 135.98, 139.0, 139.22, 146.65, 146.69, 153.58, 154.37, 155.68,
155.92, 160.64, 171.24, 171.3, 186.22, 213.81.

MS (MALDI-TOF): C\textsubscript{44}H\textsubscript{47}N\textsubscript{3}O\textsubscript{18}Na; calculated. (M+Na+) 928.28, found 928.43.

HPLC Purity analysis: See ESI Figure S1
Synthesis of 2, 5-dioxopyrrolidin-1-yl 4-azidobenzoate (4a):

4-azidobenzoic acid (4) (500 mg, 3.065 mmol) was dissolved in 10 ml of DMF cooled to 0°C. To this mixture, EDC. HCl (1.2 eq., 570 mg, 3.678 mmol) was added followed by NHS (1.2 eq., 424 mg, 3.678 mmol). The reaction was stirred in the dark under N₂ at 0 °C for approximately 1 h and then at rt for 15 h. DMF was removed in vacuo. This concentrated mixture was dissolved in 30 ml of EtOAc and then extracted with water (3x 20 ml). The organic layer was dried over MgSO₄, filtered, evaporated and purified using column chromatography (100% EtOAc) to yield 97% of pale yellow colored product.

\(^1\)H NMR (DMSO, 400 MHz):

\[ \delta = 8.11 \text{ (d, 2H, Ar-H, } J = 8.8 \text{ Hz)}, 7.36 \text{ (d, 2H, Ar-H, } J = 8.8 \text{ Hz)}, 2.90 \text{ (s, CH₂, 4H).} \]

\(^13\)C NMR (DMSO, 100 MHz):

\[ \delta = 25.48, 120.06, 120.46, 131.98, 146.80, 161.06, 170.32. \]

MS (MALDI-TOF): C₁₁H₈N₄O₄Na; calculated. (M+Na)\(^+\) 283.04, found: 283.28
Synthesis of 5-((2-(4-azidobenzamido)ethyl)amino)naphthalene-1-sulfonic acid (5):

To a stirred solution of 4a (50 mg, 0.192 mmol, 1.1 eq.) in dry DMF (10 ml) was added 
EtN(iPr)₂ (46 μL, 1.5 eq., 0.262 mmol) followed by EDANS (47 mg, 1 eq., 0.174 mmol). The 
turbid, brown solution was stirred at rt for 10 h under nitrogen. The solvent was then removed 
under low vacuum. To the resulting brown, thick oil, 30 ml of CH₂Cl₂ was added and washed 
with water (3x20 ml). The organic layer was dried over Na₂SO₄, filtered and the solvent was 
removed and purified using column chromatography (silica/15:85 MeOH: CHCl₃) to yield 46 mg 
(64%) of a brown solid.

¹H NMR (DMSO, 400 MHz):

δ = 8.72 (t, 1H, -CONH-, J = 6.0 Hz), 8.07 (d, 2H, Ar -H, J = 8.4 Hz), 7.88 (m, 3H, Ar-H), 7.27 
(dd, 2H, -CH₂, J₁₂ = 7.2 Hz, J₁₃ = 8.4 Hz), 7.21 (dd, 2H, -CH₂, J₁₂ = 8.0 Hz, J₁₃ = 7.6 Hz), 7.14 
(m, 2H, Ar-H), 6.58 (d, 1H, Ar-H, J = 7.2 Hz), 3.54 (m, 2H, -CH₂), 2.99 (m, 2H, -CH₂-).

¹³C NMR (DMSO, 100 MHz):

δ = 25.20, 45.6, 102.7, 115.6, 118.9, 122.3, 126.2, 129.1, 130.1, 130.9, 142.2, 143.71, 143.9, 
165.8, 172.7.

MS (MALDI-TOF): C₁₉H₁₇N₅O₄SNa; calculated. (M+Na)⁺ 434.09, found: 434.69
Synthesis of photocaged-cell impermeable dox. conjugate (6):

Compounds 3 (50 mg, 0.055 mmol, 1eq.) and 5 (21.7 mg, 0.05 mmol, 1eq.) were stirred in 1:1 mixture of water and DMSO (4 mL each). 100 µL (1 mg, 0.005 mmol, 0.1 eq.) of freshly prepared aqueous solution of sodium ascorbate (10 mg/mL) was added to the reaction, followed by 10 µL (0.125 mg, 0.01 eq., 0.0005 mmol) of freshly prepared aqueous solution of copper (II) sulfate pentahydrate (12.5 mg/mL) was added and finally 2.65 mg (0.1 eq., 0.005 mmol) of TBTA was added. The red colored heterogenous mixture was stirred vigorously until the reaction was completed (~24h) as judged by TLC (1: 4 CH₃OH/CHCl₃). The reaction mixture was concentrated in vacuo, filtered and the resulting solution was purified on RP-HPLC using isocratic elution of 20 % organic eluent (0.1 % TFA in acetonitrile) for 5 minutes and then increasing the acetonitrile fraction in gradient fashion from 20 % to 70% over 30 minutes. The fractions (3mL each) were collected into tubes containing 500 µL of ammonium bicarbonate (2 mg/mL) solution to quench the TFA. The two diastereomers eluted at 31.25 and 32.28 min (See ESI figure S2). The fractions containing the diastereomers were evaporated leaving a red solid (30 mg, 41%). One of the diastereomers was isolated for proton NMR analysis.
$^1$H NMR (DMSO, 400 MHz):

$\delta$ =14.06 (s, 1H), 13.29 ( s,1H), 8.85 (t, 1H, $J = 3.6$ Hz), 8.72 (s, 1H), 8.44 (t, 1H, $J = 5.2$ Hz), 8.12 (m, 1H), 8.08 (d, 2H, $J = 8.8$ Hz), 8.02 (d, 2H, $J = 8.8$ Hz), 7.91- 7.94 (m, 2H), 7.66-7.68 (m, 1H), 7.54 (s, 1H), 7.24 - 7.34 (m, 3H), 7.15 (s,1H), 7.0- 7.02 ( m, 1H), 6.64 ( d, 1H, $J = 7.6$ Hz), 6.05 (m, 1H), 5.41( m, 2H), 5.22 ( s, 1H), 4.95 (m, 1H), 4.54 (s, 2H), 4.40 (m, 2H), 4.06-4.11 (m, 3H), 4.00 (s, 3H), 3.92 (s, 3H), 3.61 – 3.63 (m, 3H), 2.98 (m, 2H), 2.67 (m, 1H), 2.54 (m, 1H), 2.32(m, 2H), 2.15 (m, 2H), 1.98 ( m, 2H), 1.84( m, 2H), 1.45 (d, 3H, $J = 6.4$ Hz), 1.09 (m, 3H).

HRMS (ESI) C$_{63}$H$_{63}$N$_8$O$_{22}$S; Calculated (M$^-$) =1315.378; Observed=1315.343  (See ESI Fig S7)

HPLC purity analysis: see ESI Fig S2

Photolytic release of doxorubicin from drug conjugate. A solution (0.065 mM in 1 mL) of compound 6 in PBS was placed in a 60x15 mm well plastic dish placed on ice. The solution was irradiated and 70$\mu$L was removed at various time points (0.5, 1, 2, 4, 8, 15, 20, and 30 minutes). Each assay was analyzed on RP-HPLC using isocratic elution of 20 % organic eluent ( 0.1 % TFA in acetonitrile) for 5 minutes and then using gradient elution from 20 % to 70% for 30 minutes and was monitored at $\lambda_{abs}$= 480 nm. (See ESI figure S3).

Illumination Time-Dependant Toxicity. JH-EsoAd1 cells were treated with media alone, media supplemented with EDANS (20 $\mu$M), or media supplemented with Dox-EDANS (9) (20 $\mu$M) in a 96 well clear-bottom, opaque plate. Cells were exposed to light (9 mW/cm$^2$) for various times (0-40 min) regulated by an aluminum foil mask. During the experiment the plate was kept in ice to reduce the heating by the UV lamp. After the lamp was turned off, the plate
was covered with foil and placed at room temperature for an additional 80 min. The media was removed, and the cells were thoroughly washed with media (3x); then 200 μL of fresh media was added to each well. The plates were incubated for 72 hours at 37 °C, following which the media was removed and 100 μL of MTT (2mg/ml) in phosphate buffered saline (PBS) was added to each well. After 3 hours, the MTT solution was removed and replaced with 100 μL of DMSO. The absorbance at 562 nm was analyzed via a plate reader.

**Concentration Dependent Cell Viability Studies:** JH-EsoAd1 cells were treated with media supplemented with various concentrations of doxorubicin (0-6.4 μM) or Dox-EDANS (6) (0-16 μM) in a 96 well clear-bottom, opaque plate. Control wells were covered with aluminum foil while the remaining wells were exposed UV light (9 mW/cm²) for 20 min. The plate was kept in ice during light exposure in order to minimize the heating. After irradiation, the plate was kept at rt in the dark for another 100 minutes. The cells were washed and treated as above (time dependent toxicity experiments) to determine viability via the MTT assay.

**Flow cytometry analysis of permeability.** JH-EsoAd1 cells were seeded at 150,000 cells/well in 1.5 mL of media in 6 well plates. The cells were treated with media alone or media supplemented with 10 μM of EDANS-Dox (6). The plate was placed on ice, and the light treated cells were exposed to UV light (9 mW/cm²) for 20 minutes. Following the light exposure the plates were kept at room temperature for 100 minutes. Then, the cells were washed with sterile PBS and trypsinized. For quantification of Dox fluorescence, treated cells were trypsinized, harvested, and washed with PBS at approximately 1x10⁶ cells/mL. Pellets were fixed for 15 min at 37 ºC with 3% paraformaldehyde in PBS. Samples were washed thoroughly with PBS, resuspended in PBS and analyzed by flow cytometry.
Confocal microscopic analysis of permeability. JH-EsoAd1 cells were seeded at 30,000 cells/well on 4 well glass chamber slides, and were treated as above (Flow cytometry analysis). The cells were treated with media alone or media supplemented with 10 μM of EDANS-Dox (6). The plate was placed on ice, and the light treated cells were exposed to UV light (9 mW/cm²) for 20 minutes. Following the light exposure the plates were kept at room temperature for 100 minutes. The cells were washed extensively with PBS and fixed for 15 min at 37°C with 3% paraformaldehyde in PBS. Slides were rinsed, dried and mounted under coverslips using Vectashield mounting media (Vector Laboratories, Inc.).
Supplementary Figures and Table

Figure S1: RP- HPLC analysis of (3) at using detection at 480 nm. The two diastereomers have retention times of 35.8 and 36.9 min.

Figure S2: RP- HPLC analysis of EDANS-Dox (6), using absorbance at 480 nm. The two diastereomers have retention times of 31.25 and 32.28 min.
**Figure S3.** a) Release of free dox with upon irradiation over time. A solution of compound 6 was irradiated. Samples were removed at various time points (0.5, 1, 2, 4, 8, 15, 20, and 30 min) and were analyzed by RP-HPLC at $\lambda = 480$ nm. The compound in the dark ($t = 0$) and free doxorubicin are included as standards. b) Graph representing increase in intensity of integrated peak area of doxorubicin in the above time dependent photolysis experiment.
Figure S4. Flow cytometry analysis of EDANS-Dox with JH-EsoAd1 cells in the dark and upon illumination (9.0 mW/cm²) for 20 min. Left Panel: Representative dot plot of the relative fluorescence intensity of EDANS-DOX in Light or Dark. The gate shown was used to derive the % Fluorescence data shown in the right panel. Right Panel: Quantification of DOX fluorescence for the indicated treatment conditions. Data are representative of two independent experiments, N=9.
**Figure S5.** Effect of light and EDANS on cell survival. JHEsoAd1 cells were treated with EDANS in the dark (blue diamonds), EDANS + light (open squares) (9 mW/cm²) or light alone (black diamonds) for the specified times. Cell survival was determined using the MTT assay. Each data point was taken from a minimum of six replicate experiments. Error bars are omitted for clarity.
Figure S6. Concentration-dependent toxicity of compounds on JH-EsoAd1 cells. % cell viability was plotted for JH-EsoAD1 cells at various concentrations of EDANS-Dox in the dark (open squares) or with light (black diamonds, black line), and unconjugated Dox in the dark (red circles, red line) or with light (blue diamonds, blue line). Experiments were performed in at least six replicates. Error bars are omitted for clarity. The lines are plots of the curve fits of the relevant data to the equation: $Y = M1 + (M2-M1)/(1+X/M3)$. The constant M3 describes the IC$_{50}$ value.
Figure S7: HRMS of Photocaged doxorubicin conjugate 6

Table S1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ value (μM)</th>
<th>Std. Error</th>
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</thead>
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<tr>
<td>EDANS-Dox No Light</td>
<td>No toxicity</td>
<td></td>
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<tr>
<td>EDANS-Dox + Light</td>
<td>1.6</td>
<td>1.0</td>
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<tr>
<td>Doxorubicin No light</td>
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</tr>
<tr>
<td>Doxorubicin + light</td>
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<td>0.14</td>
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</table>
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Electronic Supplementary Material (ESI) for Chemical Communications

$S = $ solvent peak

$S = $ solvent peak
3

4-NP = 4-nitrophenol

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4-NP = 4-nitrophenol