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Synthesis and photolytic evaluation of a nitroindoline-caged glycine with a side chain of high negative charge for use in neuroscience☆

George Papageorgiou [a,*], Marco Beato [b], David Ogden [c]

[a] MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[b] Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London WC1E 6BT, UK
[c] CNRS Unité 8118, Université Paris Descartes, 75006 Paris, France

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ABSTRACT

A photolabile precursor of the neuroinhibitory amino acid glycine has been synthesised with two phosphate groups attached to the indoline nucleus at a 4-alkoxy substituent. In common with the photochemical properties of other 1-acyl-7-nitroindolines, this releases glycine on a sub-μs time scale upon irradiation with near-UV light. The synthetic route previously developed for the preparation of the GABA analogue required some modifications because of the greater hydrolytic sensitivity of the glycine compound. The phosphorylation method used here could be beneficial to the synthesis of other nitroindoline-caged amino acids, especially the related caged GABA derivative. Glycine released by laser photolysis on spinal cord neurons generated fast-rising responses and the pharmacological properties of the reagent are such that it is useful for physiological experiments.

1. Introduction

The technique of liberating biologically active compounds from photolabile precursors, upon brief irradiation with near-UV light is a valuable tool for studies of biological processes. The method relies on the availability of suitable reagents, widely known as 'caged' compounds and their syntheses and applications are described in reviews.[1] These reagents must satisfy a number of prerequisites such as solubility and stability in aqueous media, high purity and pharmacological inertness prior to photolysis, rapid and efficient photolysis at neutral pH followed by fast dark reactions after photolysis, and no release of toxic by-products after photocleavage. Failure to fulfil any one of the above criteria usually renders a caged reagent inadequate for application in biological systems.

During the last decade we have produced a number of 7-nitroindoline-caged neuroactive amino acids, including L-glutamates **1**[2] and **2**,[3] γ-aminobutyrate (GABA) **3** and glycine **4**,[4] L-aspartate **5**,[5] and L-alanine **6**,[6] which have been applied in various biological systems. Detailed pharmacological evaluation has found that caged glutamates **1** and **2** do not bind to glutamergic receptors prior to photolysis and are therefore suitable reagents for investigations of synaptic transmission.[4,5,7] They fulfil all the above criteria to a satisfactory extent, they are now commercially available and have been extensively applied in neuroscience.[8] In contrast, caged GABA **3** and caged glycine **4** were found to bind to their respective ionotropic GABA or glycine receptors on neuronal cells prior to photolysis, blunting the response to the photolytically released amino acid.[4,7] A separate investigation has indicated that neither caged glycine **4** nor caged L-aspartate **5** inhibit the NMDA receptor, suggesting that reagent **4** might have useful application in studies of the effects of co-agonist binding to NMDA receptors.[5] Photolytic release of L-alanine from **6** has enabled the observation of alanine-induced pre-steady-state currents of three different neutral amino acid transporters.[6]

Photolysis of these reagents in aqueous solution proceeds with stoichiometric release of the amino acid, accompanied by the corresponding nitrosoindole by-product as shown in Scheme 1. A study of the photolysis mechanism[9] of 1-acyl-7-nitroindolines in aqueous solution determined the release rate of the amino acid as $\sim 5\times10^{6}\ s^{-1}$ at ambient temperature, corresponding to a half-life of ~150 ns, which is sufficiently rapid for mimicking biological processes.

The advantageous photochemical properties and hydrolytic stability of 1-acyl-7-nitroindolines motivated us to seek means of eliminating or at least suppressing the adverse pharmacological properties of **3** and we have previously reported the synthesis and photochemical evaluation of the modified caged GABA reagent **7**.[10] The incorporation of a high concentration of negative charge close to the nitroindoline nucleus did not affect its photochemical properties. Furthermore, the structure retained the advantages of hydrolytic stability of the cage and its fast and efficient

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* Corresponding author. Tel.: +44 20 8816 2407; fax: +44 20 8906 4477; e-mail address: gpapage@nimr.mrc.ac.uk (G. Papageorgiou).

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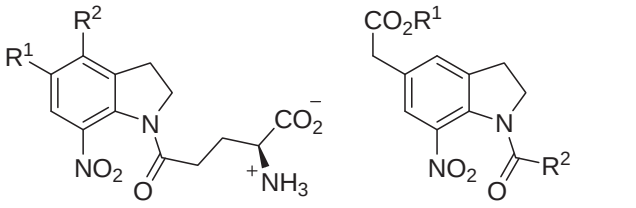
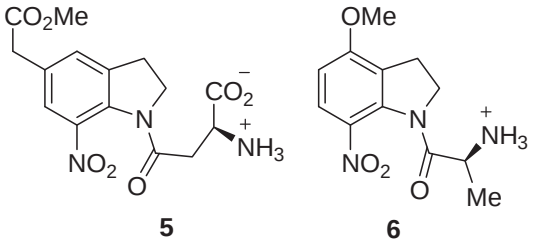


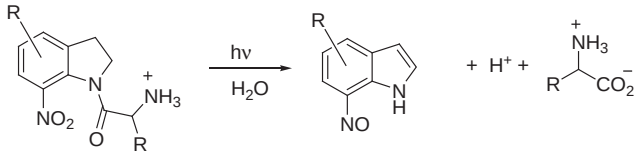
1 $R^1 = CH_2CO_2Me$, $R^2 = H$ **3** $R^1 = Me$, $R^2 = (CH_2)_3\overset{+}{N}H_3$

2 $R^1 = H$, $R^2 = OMe$ **4** $R^1 = H$, $R^2 = CH_2\overset{+}{N}H_3$



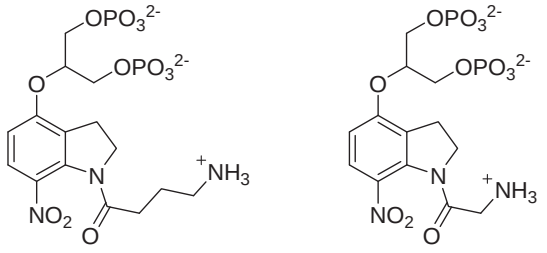
5 **6**

photorelease at near-UV and 405 nm wavelengths, but brought about a large reduction in the interaction of **7** with $GABA_A$ receptors.[11a] Further detailed neurophysiological investigations[11b] revealed the existence of a new class of miniature currents ('preminis') in developing interneurons that reflect the activation of presynaptic $GABA_A$ receptors following random vesicular release.



Scheme 1. Photocleavage reaction of 7-nitroindoline-caged amino acids in aqueous solution.

The successful reduction of adverse pharmacological properties of **7** prior to photolysis opened the possibility that the same strategy might be applied to the glycine analogue **8**. Various caged glycine derivatives have been reported by other workers[12] but have suffered from problems of instability or inadequate photolytic efficiency. We had anticipated that synthesis of the diphosphate nitroindoline (DPNI)-glycine **8** would closely follow the route described for **7**. In the event, a number of modifications were required, in part because of greater hydrolytic sensitivity of the amide bond in the glycine series of precursors. In addition, the method for introduction of the two phosphate groups was substantially improved over that previously described in the synthesis of **7**. In the course of developing the synthesis, we also prepared the 4-methoxy compound **15** and in the process obtained some further insight into side reactions that occurred during nitration reactions of *N*-acylindolines.



7 **8**

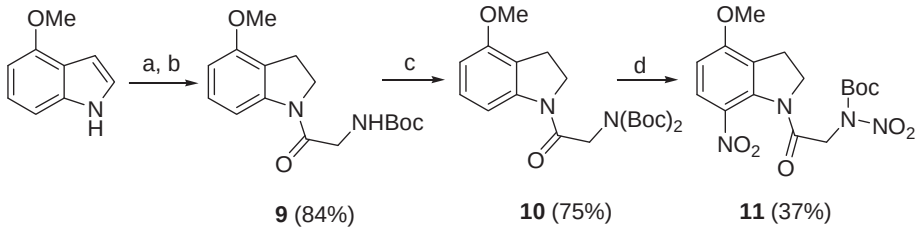
2. Results and discussion

In our previous investigations[10] of a synthetic route to **7**, we had found that di-Boc protection of the amino group in a GABA precursor had been ineffective in avoiding side reactions at this centre, although the same protection strategy had been effective for amino groups in precursors to 4-methoxy-7-nitroindoline derivatives of L-glutamate,[13] and L-alanine.[6] For the eventual successful synthesis of **7**, the problem was avoided by masking the amino group as an azide that was converted to an amine at a late stage of the synthesis. Nevertheless, in view of the successful application of the di-Boc protection strategy for synthesis of L-alanine, we felt it was worthwhile to test the same methodology here. Using the 4-methoxy system, the route shown in Scheme 2 gave the di-Boc precursor **10**. Homogeneous nitration, as used by us previously for related nitrations,[3b,6,13] gave a mixture in which the major component was the *N*-nitro species **11**, isolated as a crystalline solid and characterised by ^1H NMR spectroscopy and elemental analysis (see Supplementary data). Although 5- and 7-mononitro isomers were also present in the reaction products, the substantial formation of **11** made this route unacceptable. It now appears that both steric and electronic effects are important in the ability of the double Boc protection to limit unwanted N-nitration, and we therefore returned to the azide strategy used for **7**.

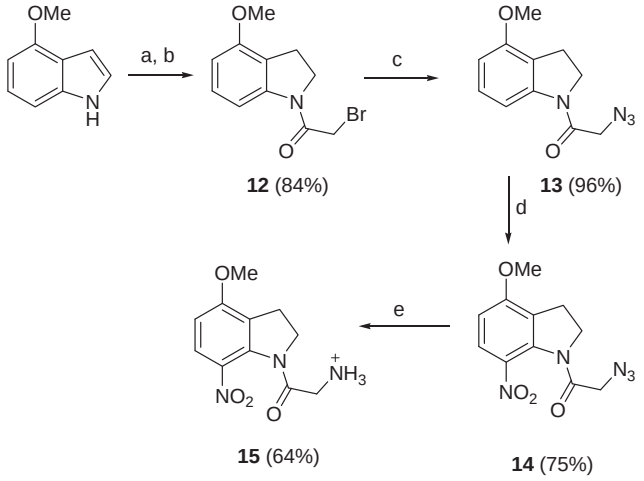
Given the somewhat unpredictable outcome of results with only minor changes in structure, we first tested the azide route by preparing the methoxynitroindoline-caged glycine **15**, as shown in Scheme 3, both to validate the individual steps and to have a sample of **15** available for pharmacological testing in comparison with our intended target compound **8**. Only two points of Scheme 3 deserve specific comment. First, the nitration of the azidoacetylindoline **13** was carried out using a minor modification of a reported procedure[14] ($NaNO_3$ in place of $LiNO_3$) in which scandium triflate acts as a catalyst for nitration. In practice, for this substrate there was no advantage in yield or regioselectivity over our previous claycop-mediated nitration procedure,[3b] but the scandium triflate procedure was investigated as an alternative because of difficulties encountered during the synthesis of **8** (see below) and because we had previously found that the claycop procedure was ineffective with indolines bearing a bulky substituent at the 4-position.[10] The second point of note relates to the Staudinger reduction of the azide **14** that was carried out in aq DMF, as had been done at the final stage in synthesis of **8**. In the latter case the initial product isolated had been an iminophosphorane, which required mild but prolonged acid treatment (pH 1 at rt overnight) to release the required amine.[10] In contrast, the reduction of **14** under the same conditions gave the amine directly upon work-up of the reaction mixture. As discussed below, the susceptibility to hydrolysis of the initial iminophosphorane product is remarkably idiosyncratic.

With preparation of **15** readily achieved, we returned to the synthesis of **8** and, following similar methods to those in Scheme 3, readily obtained the azide **19** (Scheme 4). However, upon very brief alkaline treatment (25 mM NaOH for 2 min), which was intended to remove the acetate groups, a mixture of products was detected by TLC and UV–vis spectroscopy that indicated significant hydrolysis of the amide linkage had taken place. This contrasts with experience in the synthesis of the caged GABA **7**, where hydrolysis of the corresponding acetates was readily achieved under very similar conditions, without detectable amide hydrolysis. Electron-withdrawal by the α-azido substituent evidently makes the azidoacetyl amide bond much more sensitive to base hydrolysis and we subsequently found that the amide bond was ~10% hydrolysed at pH 7.0 overnight at rt (see details in Supplementary data).

To avoid the problem of amide hydrolysis, we instead carried out the hydrolysis step before nitration, since the amide bond in the non-nitrated indoline was expected to be much more stable. The

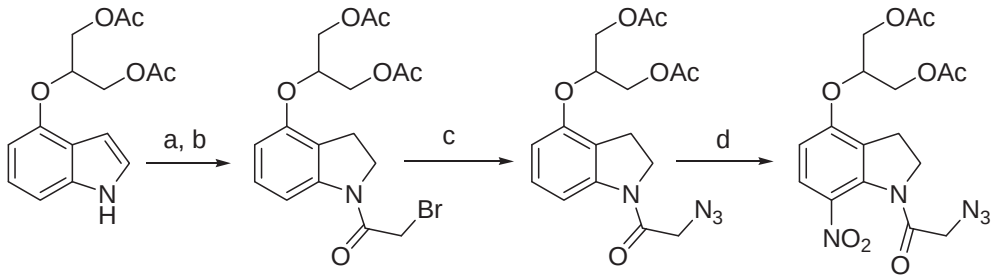


Scheme 2. Reagents: (a) $NaBH_3CN$–AcOH; (b) EDC–HO_2CCH_2NHBoc–MeCN; (c) $(Boc)_2O$–Et_3N–DMAP–CH_2Cl_2; (d) $Cu(NO_3)_2$–Ac_2O–CH_2Cl_2.

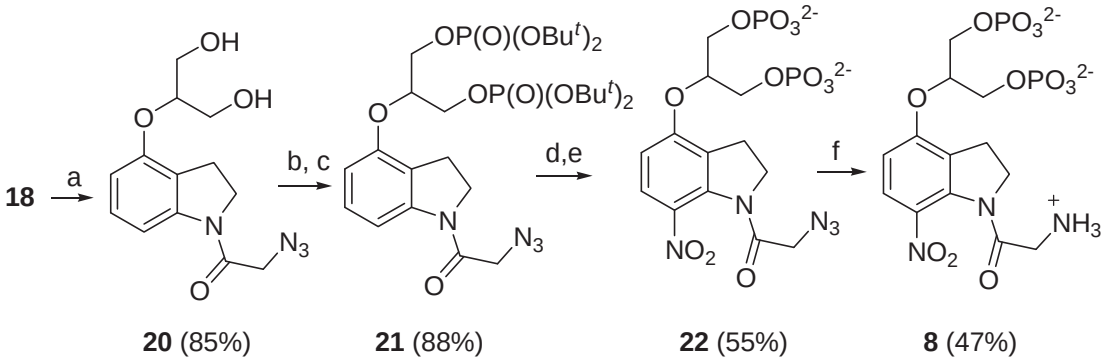


Scheme 3. Reagents: (a) $NaBH_3CN$–AcOH; (b) EDC–$BrCH_2CO_2H$–MeCN; (c) NaN_3–DMF; (d) $NaNO_3$–Ac_2O–$Sc(OTf)_3$–MeCN; (e) PPh_3–aq DMF.

diacetate **18** was readily hydrolysed to the diol **20** (Scheme 5) and we intended to introduce the two phosphate groups (in protected form) at this stage. Phosphorylation of a related diol in the caged GABA synthesis using phosphoramidite chemistry had been problematic because of low solubility of the diol in THF. A change to dichloromethane solvent with pyridinium trifluoroacetate (PyTFA) as the phosphitylation catalyst[15] followed by oxidation with m-CPBA gave the bis(di-*tert*-butyl phosphate) **21** in excellent yield. Nitration of the protected bis-phosphate **21** was complicated by the presence of the acid-sensitive *tert*-butyl esters, which underwent partial deprotection under any of the conditions tried. After numerous trial reactions, it was found that the nitration of diphosphate **21** was best achieved with a mixture of acetic anhydride, sodium nitrate and a 4-fold excess of trichloroacetic acid (relative to the amount of $NaNO_3$). As noted above, partial deprotection of the phosphate groups had taken place, indicated by the fact that the compound had become water-soluble and behaved as an anion on analytical anion-exchange HPLC (see Experimental section). Rather than characterise the partially deprotected product, although it appeared to be principally a single species, we felt it



Scheme 4. Reagents: (a) $NaBH_3CN$–AcOH; (b) EDC–$BrCH_2CO_2H$–MeCN; (c) NaN_3–DMF; (d) $Cu(NO_3)_2$–Ac_2O–CH_2Cl_2.



Scheme 5. Reagents: (a) NaOH–aq MeOH; (b) $Et_2NP(O^tBu)_2$–PyTFA–CH_2Cl_2; (c) MCPBA; (d) $NaNO_3$–Ac_2O–CCl_3CO_2H–MeCN; (e) TFA; (f) TCEP–aq DMF.

was more convenient to remove the remaining *tert*-butyl groups but found that prior removal of excess nitrate salt from the reaction mixture was required before treatment with TFA. Without such removal, treatment of the freeze-dried reaction mixture with TFA resulted in an intractable dark mixture, and efforts to quench the excess nitrate by addition of reactive electrophiles prior to work-up were also ineffective. Thus the crude water-soluble reaction mixture was subjected to preparative reverse-phase HPLC and the isolated nitroindoline product, after lyophilisation, was successfully deprotected with TFA to give **22** in 55% yield.

With **22** available, completion of the synthesis required only reduction of the azide group, but Staudinger reaction using either triphenylphosphine or tri-*n*-butylphosphine in aq DMF failed to yield pure **8** even after prolonged mild acidic treatment of the reaction mixture. It was evident from HPLC data (not shown) that an iminophosphorane was formed, as in the case of the related caged GABA synthesis,[10] but it was much more resistant to hydrolysis. This was particularly surprising since reduction of **14** under the same conditions had given the amine **15** directly upon mild acidic work-up (see above), implying ready hydrolysis of the iminophosphorane in that case. However, reduction of the azide **22** was successfully accomplished using the water-soluble tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to give **8** in 47% yield after purification by anion-exchange chromatography, precipitation as its barium salt and reconversion to the sodium salt.

The photochemistry of both **8** and its model analogue **15** was similar to that of other *N*-acyl-7-nitroindolines. Continuous irradiation of separate solutions of **8** and **15**, monitored by UV–vis spectroscopy, as described previously,[2,3a,10] showed progressive decrease of each starting compound and accumulation of the nitrosoindole chromophore. Clear isosbestic points were formed as photolysis progressed indicating a clean conversion (see Supplementary data). Photolysis of **8** was also monitored by HPLC and released glycine was measured by amino acid analysis, as described for related compounds.[2,3a] Glycine recovery was in the range 68–69%. This was significantly below the essentially stoichiometric release reported for other 7-nitroindoline-caged amino acids and appears inconsistent with the clean spectroscopic data for the progressive photolysis but extensive investigations (data not shown) have not resolved this matter.

2.1. Neurophysiological recordings upon glycine release by near-UV laser photolysis

Glycine is a major inhibitory fast neurotransmitter in the mammalian spinal cord. To test the photorelease of glycine from DPNI-glycine **8** and the effects of **8** itself upon neuronal preparations, experiments were done with laser photolysis in spinal cord slices prepared from neonatal rats. Whole cell patch clamp recordings were made under conditions of high intracellular chloride ion concentration, generating inward membrane current in response to activation of glycine receptors by glycine photoreleased from **8**. The morphology and identity of recorded neurons were obtained by epifluorescence imaging of the fluorophore Alexa 488 included in the intracellular solution in the whole cell-recording pipette. Photolysis was with laser pulses at 405 nm usually of 0.2 ms duration as described previously.[11a] The laser spot diameter was 1 μm in the focal plane and could be directed to the soma or neurites of recorded neurons with sub-micron accuracy. The experimental methods are described in Supplementary data.

Responses recorded in a motoneuron to concentrations of glycine released at different laser intensities in 0.2 ms pulses from DPNI-glycine **8**, present at 2 mM, are shown in Fig. 1A. The amplitude of the response increases with laser intensity (0.4–8.1 mW) up to the point of saturation, as shown in the bar chart of Fig. 1D. The 20–80% rise times increased from 1.2±0.2 to 2.8±0.5 ms with increasing intensity of the pulse. For comparison, the rise time of spontaneous post-synaptic currents (shown in Fig. 1B) is in the 0.3–1.1 ms range, over a population of 12 cells. As the laser intensity was increased, also the decay time-constant increased, from 8.1±0.4 to 28.0±6.1 ms, compared with corresponding values for spontaneous currents of between 4.2 and 9.1 ms. The slower rise and fall of the responses to photolytically released glycine is apparent in the normalised and superimposed traces of Fig. 1C. For comparison, an average of spontaneous post-synaptic currents is also shown. The slower kinetics may be attributed to the larger

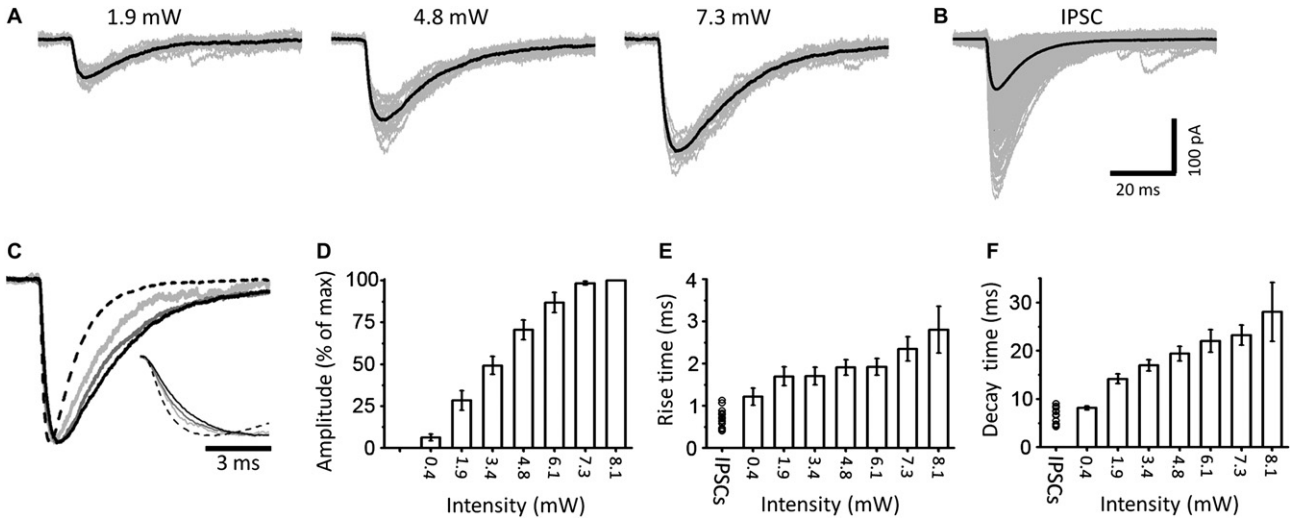


Fig. 1. Responses in spinal motorneurons under whole cell voltage clamp to laser-evoked photolysis of extracellular 2 mM DPNI-glycine **8** in a 1 μm focal spot. Negative deflections show inward membrane current due to Cl^- ion flux through receptor ion channels activated by glycine binding. (A) Increasing intensity of the laser pulse (duration 0.2 ms) generates progressively larger amplitudes of response. Grey traces are superimposed individual events; black traces are the mean at each intensity. Laser power in mW is given at the objective, with 2 mM of **8** present in the bath 50% of the light is absorbed by inner filtering. (B) For comparison spontaneous glycine synaptic responses and their mean are shown on the same scale. (C) The time course of normalised responses to low (light grey), medium and high intensity (black) pulses are compared with synaptic responses (dashed line) on the same time scale. The inset shows a time scale expansion of the rise times of the averaged response (continuous lines) and of spontaneous events (dashed line). (D, E, F) Summary bar charts of data from 12 neurons of the peak amplitude (D), the 20–80% rise time (E) and the exponential time-constant of response decline (F). In panels (E) and (F) the left column is data for spontaneous IPSCs due to synaptic glycine release in the same neurons, other columns show responses to glycine released by different laser intensities.

volume into which glycine is released by laser spot photolysis compared with the volume of synaptic release.

Direct interference between uncaged DPNI-glycine **8** and glycine receptors was tested by measuring the amplitude of spontaneous synaptic currents in control and in the presence of 3 mM DPNI-glycine. The mean event amplitudes as well as their rise times did not change in the two conditions (see Supplementary data Fig. S2 and text). However, following repetitive uncaging on the same spot, a rundown of the light evoked current was consistently observed (Fig. S2 and text). The origin of this rundown will require further investigations, but it could be associated with the observed incomplete stoichiometry obtained on photolysis of DPNI-glycine.

3. Conclusions

The synthesis of **8** was successfully accomplished but, despite its similarity to the previously described caged GABA **7**, significant modification of the previously established synthetic route was required. The unexpected reactivity of the densely functionalised intermediate compounds was surmounted by employing more effective reagents and appropriate reaction conditions. The controlled nitration of indolines in synthesis of these caged compounds remains subject to a need for individual optimisation, while the improved phosphorylation method could usefully be applied to a better synthesis of the caged GABA **7**.

Release of glycine on spinal cord neurons by localised laser photolysis of **8** showed fast activation of glycinergic currents with time course in the millisecond range. Although slower than glycine mediated synaptic currents recorded in the same neurons, the kinetics of activation by photoreleased glycine are nonetheless fast enough to be useful in experiments to determine the distribution of glycine sensitivity in neuronal compartments and to yield kinetic data of glycine receptor function at synaptic sites in situ. A further appealing feature of **8** is that, contrary to the block of GABA (γ-aminobutyric acid) responses seen with the caged GABA analogue DPNI-GABA **7**, DPNI-glycine does not interact with the glycine receptors. The origin of the rundown of responses seen with multiple pulses of DPNI-glycine photolysis applied consecutively to the same sites was not elucidated in the experiments described here. The present synthesis has provided enough material for further pharmacological characterisation and these studies will be reported elsewhere in due course.

4. Experimental

4.1. General

^{1}H NMR spectra were determined on Varian Inova 600 MHz or Varian Inova 800 MHz spectrometer in $CDCl_3$ solution with TMS as internal reference, unless otherwise specified. Elemental analyses were carried out by MEDAC Ltd., Surrey, UK. Electrospray mass spectra were recorded at the School of Pharmacy, University of London. Merck 9385 silica gel was used for flash chromatography. Analytical HPLC was performed on a 250×4 mm Merck Lichrospher RP8 column or a 125×4 mm Whatman Partisphere SAX column. Flow rate was 1.5 mL min^{-1} with either column. Preparative HPLC was carried out on a 2×30 cm column (Waters C_{18} packing, Cat. No. 20594) at 2 mL min^{-1} flow rate. Details of mobile phases are given at relevant points in the text. Preparative anion-exchange chromatography used a column of DEAE-cellulose (2×20 cm). Detection for all analytical and preparative chromatography was at 254 nm. Organic solvents were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Hexanes (bp 40–60 °C) were redistilled before use. Photolysis experiments were performed in a Rayonet RPR-100 photochemical reactor fitted with 16×350 nm lamps.

4.2. 1-(2-Bromoacetyl)-4-(1,3-diacetoxypropan-2-yloxy) indoline (17)

A solution of 4-(1,3-diacetoxypropan-2-yloxy)indoline (2.93 g, 10 mmol; prepared from **16** as previously described[10]) in dry MeCN (70 mL) was treated with 2-bromoacetic acid (2.08 g, 15 mmol), followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.68 g, 14 mmol) and the mixture was stirred at rt under nitrogen overnight. The solvent was evaporated and the residue was taken up in EtOAc (80 mL), washed with dilute aq HCl, saturated aq $NaHCO_3$ and brine, dried and evaporated to pale crystals. Recrystallisation (EtOAc–hexanes) followed by flash chromatography [EtOAc–hexanes (2:3)] of the mother liquor gave **17** (3.17 g, 77%) as white crystals, mp 77–79 °C (EtOAc–hexanes); ^{1}H NMR (600 MHz) δ 7.87 (d, J=8.2 Hz, 1H), 7.19 (t, J=8.2 Hz, 1H), 6.73 (d, J=8.2 Hz, 1H), 4.71 (quintet, J=5.2 Hz, 1H), 4.34 (dd, J=5.7, 11.9 Hz, 2H), 4.29 (dd, J=4.9, 11.9 Hz, 2H), 4.21 and 4.18 (2×t, J=8.5 Hz, 2H, rotamers), 4.16 (s, 2H), 3.16 (t, J=8.5 Hz, 2H), 2.07 (s, 6H). Anal. Calcd for $C_{17}H_{20}NO_6Br$: C, 49.29; H, 4.87; N, 3.38. Found: C, 49.53; H, 4.67; N, 3.63.

4.3. 1-(2-Azidoacetyl)-4-(1,3-diacetoxypropan-2-yloxy) indoline (18)

NaN_3 (0.97 g, 15 mmol) was added to a solution of **17** (2.07 g, 5 mmol) in dry DMF (40 mL) and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure and the residue was diluted with EtOAc (70 mL), washed with water and brine, dried and evaporated to give **18** as white crystals (1.69 g, 90%), mp 64–65 °C (Et_2O–hexanes); ^{1}H NMR (600 MHz) δ 7.89 (d, J=8.1 Hz, 1H), 7.19 (t, J=8.1 Hz, 1H), 6.72 (d, J=8.1 Hz, 1H), 4.71 (quintet, J=5.2 Hz, 1H), 4.34 (dd, J=5.8, 11.9 Hz, 2H), 4.29 (dd, J=4.7, 11.9 Hz, 2H), 4.00 (t, J=8.2 Hz, 2H), 3.97 (s, 2H), 3.14 (t, J=8.2 Hz, 2H), 2.06 (s, 6H). Anal. Calcd for $C_{17}H_{20}N_4O_6$: C, 54.25; H, 5.36; N, 14.89. Found: C, 54.29; H, 5.52; N, 14.68.

4.4. 1-(2-Azidoacetyl)-4-(1,3-diacetoxypropan-2-yloxy)-7-nitroindoline (19)

To a solution of **18** (489 mg, 1.35 mmol) in a mixture of CH_2Cl_2 (13.5 mL) and acetic anhydride (27 mL) was added copper nitrate hemipentahydrate (365 mg, 1.75 mmol) and the mixture was stirred at rt overnight. The solution was concentrated and the residue was re-evaporated from toluene, diluted with EtOAc (50 mL) and washed with saturated aq $NaHCO_3$ and brine, dried and evaporated to brown viscous oil. Flash chromatography [EtOAc–hexanes (1:1)], followed by trituration with ether, gave **19** (407 mg, 72%) as yellow crystals, mp 90–91 °C (EtOAc–hexanes); ^{1}H NMR (800 MHz) δ 7.80 (d, J=9.0 Hz, 1H), 6.84 (d, J=9.0 Hz, 1H), 4.82 (quintet, J=5.3 Hz, 1H), 4.36 (dd, J=5.9, 11.9 Hz, 2H), 4.31 (dd, J=4.7, 11.9 Hz, 2H), 4.22 (t, J=8.1 Hz, 2H), 4.04 (s, 2H), 3.12 (t, J=8.1 Hz, 2H), 2.07 (s, 6H). Anal. Calcd for $C_{17}H_{19}N_5O_8$: C, 48.46; H, 4.54; N, 16.61. Found: C, 48.12; H, 4.47; N, 16.37.

4.5. 1-(2-Azidoacetyl)-4-(1,3-dihydroxypropan-2-yloxy) indoline (20)

A solution of **18** (1.51 g, 4 mmol) in MeOH (80 mL), water (8 mL) and 1 M aq NaOH (9.6 mL, 9.6 mmol) was stirred at rt for 2 min, quenched with 1 M aq citric acid (19.2 mL) and concentrated to ~40 mL. The solution was diluted with water (20 mL), extracted with EtOAc (3×40 mL) and the combined organic extract was washed with saturated aq $NaHCO_3$ and brine, dried and evaporated to give **20** (1.00 g, 85%) as white crystals, mp 110–112 °C (EtOAc–hexanes); ^{1}H NMR (800 MHz; DMSO-d_6) δ 7.66 (d, J=8.1 Hz, 1H), 7.13 (t, J=8.1 Hz, 1H), 6.78 (d, J=8.1 Hz, 1H), 4.77 (t,

J=5.5 Hz, 2H), 4.26 (quintet, J=5.5 Hz, 1H), 4.22 (s, 2H), 4.00 (t, J=8.4 Hz, 2H), 3.58–3.61 (m, 2H), 3.33–3.56 (m, 2H), 3.04 (t, J=8.4 Hz, 2H). Anal. Calcd for $C_{13}H_{16}N_4O_4$: C, 53.42; H, 5.52; N, 19.16. Found: C, 53.69; H, 5.61; N, 18.80.

4.6. 1-(2-Azidoacetyl)-4-[1,3-bis(di-*tert*-butoxyphosphoryloxy)propan-2-yloxy]indoline (21)

A solution of **20** (0.58 g, 2 mmol) in dry CH_2Cl_2 (60 mL) was treated under nitrogen with pyridinium trifluoroacetate (1.16 g, 6 mmol) and di-*tert*-butyl *N*,*N*-diethylphosphoramidite (93% purity; 1.61 g, 6 mmol) and the mixture was stirred under nitrogen at rt for 20 min. The solution was cooled to 0 °C and treated dropwise with a solution of *m*-chloroperbenzoic acid (77% peracid; 2.69 g, 12 mmol) in CH_2Cl_2 (60 mL). The solution was stirred at 4 °C for 45 min, diluted with CH_2Cl_2 and washed successively with 10% aq $Na_2S_2O_5$, saturated aq $NaHCO_3$ and brine, dried and evaporated. Flash chromatography (EtOAc) gave **21** (1.19 g, 88%) as white crystals, mp 78–80 °C (Et_2O–hexanes); ^1H NMR (600 MHz) δ 7.87 (d, J=8.2 Hz, 1H), 7.18 (t, J=8.2 Hz, 1H), 6.75 (d, J=8.2 Hz, 1H), 4.70 (quintet, J=4.9 Hz, 1H), 4.18 (d, J=5.2 Hz, 2H), 4.17 (d, J=5.2 Hz, 2H), 3.98 (t, J=8.4 Hz, 2H), 3.97 (s, 2H), 3.18 (t, J=8.4 Hz, 2H), 1.47 (s, 18H), 1.45 (s, 18H). Anal. Calcd for $C_{29}H_{50}N_4O_{10}P_2$: C, 51.47; H, 7.45; N, 8.28. Found: C, 51.87; H, 7.591; N, 8.18.

4.7. 1-(2-Azidoacetyl)-4-[1,3-bis(dihydroxyphosphoryloxy)propan-2-yloxy]-7-nitroindoline (22)

To a solution of **21** (237 mg, 0.35 mmol) in dry MeCN (3.5 mL) were added acetic anhydride (357 mg, 3.5 mmol), sodium nitrate (59.5 mg, 0.7 mmol) and trichloroacetic acid (457 mg, 2.8 mmol) and the mixture was stirred under nitrogen at rt for 24 h. TLC [MeOH–EtOAc (1:19)] confirmed that all starting material was consumed. The reaction mixture was diluted with 200 mM NaOAc, pH 6.0 (70 mL), the pH was raised from 4.8 to 5.5 with 1 M aq NaOH and the solution was washed with Et_2O (3×50 mL). Analysis of the aqueous phase by reverse-phase HPLC [mobile phase 25 mM Na phosphate, pH 6.0–MeCN (25:1), t_R 5.8 min], and by anion-exchange HPLC [mobile phase 100 mM Na phosphate, pH 6.0–MeCN (5:1), t_R 5.2 min], confirmed that all starting material was nitrated but partial deprotection of phosphate esters took place. The solution was loaded onto the preparative HPLC column, which was pre-equilibrated with 200 mM NaOAc, pH 6.0, and subsequently eluted with the same buffer for further 1.5 h. The column was then washed with water and the compound began to elute after about 1 h, when the conductivity of the eluate had lowered to that of water. The fractions containing the compound were combined, concentrated to about 25 mL and lyophilised. The pale yellow residue was dissolved in TFA (15 mL) and stirred at rt for 1 h. The solvent was evaporated, the residue was dissolved in 25 mM triethylammonium phosphate, pH 6.0 (75 mL) and the pH was raised from 2.6 to 6.0 with 1 M aq NaOH. HPLC analysis as above confirmed full deprotection of the phosphate esters. The solution was loaded onto the preparative HPLC column, which was pre-equilibrated with 25 mM triethylammonium phosphate, pH 6.0, and subsequently eluted with the same buffer for further 1 h. The column was then washed with water and the compound began to elute after about 50 min, when the conductivity of the eluate had lowered to that of water. Fractions containing the compound were analysed by reverse-phase HPLC [mobile phase 100 mM Na phosphate, pH 6.0–MeCN (25:1), t_R 3.6 min], and by anion-exchange HPLC [mobile phase 100 mM Na phosphate, pH 6.0–MeCN (5:1), t_R 5.8 min], combined and concentrated. The residue was taken up in water (35.5 mL), passed through a 0.2 μm membrane and quantified by UV–vis spectroscopy to give pure **22** (5.47 mM, 194 μmol, 55%). An aliquot (0.6 mL) was exchanged to the Na^+ salt (Dowex 50, Na^+ form); ^1H NMR (600 MHz, D_2O, acetone ref.) δ 7.85 (d, J=9.0 Hz, 1H), 7.13 (d, J=9.0 Hz, 1H), 4.92 (quintet, J=4.7 Hz, 1H), 4.37 (s, 2H), 4.25 (t, J=7.8 Hz, 2H), 4.09–4.17 (m, 4H), 3.23 (t, J=7.9 Hz, 2H); LRMS (ESI): calcd for $(C_{13}H_{13}N_5O_{12}P_2+3H)^-$ 496.0, found: 495.9. The remainder of the solution was lyophilised to a pale yellow solid and used in the next step.

4.8. 1-(2-Aminoacetyl)-4-[1,3-bis(dihydroxyphosphoryloxy)propan-2-yloxy]-7-nitroindoline (8)

A solution of **22** (191 μmol) in water (3 mL) was diluted with DMF (27 mL) and treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (383 mg, 1.34 mmol) and the mixture was stirred at rt under nitrogen for 28 h. The solution was concentrated under reduced pressure and the residue was diluted with water (50 mL) and washed with EtOAc (3×40 mL). Analysis by reverse-phase HPLC [mobile phase 25 mM Na phosphate, pH 6.0–MeCN (25:1), t_R 1.8 min], and by anion-exchange HPLC [mobile phase 100 mM Na phosphate, pH 6–MeCN (5:1), 1.5 mL/min, t_R 5.2 min] confirmed complete reduction of the azide. The solution was then adjusted to pH 6.0 with 1 M aq NaOH and quantified by UV–vis spectroscopy (51 mL, 3.7 mM, 189 μmol). The solution was diluted with water (250 mL) to conductivity 5.3 mS cm^{-1} and subjected to anion-exchange chromatography using a linear gradient formed from 10 and 500 mM NaOAc, pH 6.0 (each 1000 mL). Fractions containing the product, which eluted at ~300 mM NaOAc, were analysed as above, combined (144 mL) and quantified by UV spectroscopy (0.678 mM, 98 μmol). The solution was concentrated to about 4 mL, diluted with water to 8 mL, treated with 2 M $Ba(OAc)_2$ (2 mL) and EtOH (4 mL) and allowed to stand at 4 °C overnight. The mixture was centrifuged and the supernatant was analysed by UV–vis spectroscopy (9% of original concentration, i.e., 91% precipitation). The precipitate was washed with water–EtOH (1:1) (5×15 mL) by resuspension and subsequent centrifugation after each wash cycle. The final precipitate was dissolved in water (15 mL) and mixed with Dowex 50 (Na^+ form; 4 g) for 2 h. The resin was filtered off, washed with water (20 mL) and the combined filtrates were adjusted from pH 8.4 to 6.8 with 1 M aq HCl. The filtrate was passed through a 0.2 μm membrane filter, lyophilised and the residue was dissolved in water (4 mL) and quantified by UV–vis spectroscopy to give **8** (22.3 mM, 89 μmol, 47%) as the Na^+ salt [containing a small amount of tris(2-carboxyethyl)phosphine oxide in approximately 4:1 ratio]; ^1H NMR (500 MHz, D_2O, acetone ref.) δ 8.00 and 7.86 (2×d, J=9.2 Hz, 1H, rotamers), 7.90 and 7.20 (2×d, J=9.2 Hz, 1H, rotamers), 4.89 and 4.55 (2×quintet, J=5.0 Hz, 1H, rotamers), 4.27 and 4.21 (2×t, J=7.9 Hz, 2H, rotamers), 4.14 (s, 2H), 4.00–4.07 (m, 4H), 3.47 and 3.27 (2×t, J=7.9 Hz, 2H, rotamers); LRMS (ESI): calcd for $(C_{13}H_{16}N_3O_{12}P_2+2H)^-$ 470.0, found: 470.0. Complete separation from tris(carboxyethyl)phosphine oxide was not achieved in the purification, as shown by signals in the ^1H NMR spectrum at δ 2.38–2.43 [m, P(O)($CH_2CH_2CO_2$)$_3$], 2.09–2.14 [m, P(O)($CH_2CH_2CO_2$)$_3$], with intensities corresponding to ~20 mol % of the concentration of **8**.

4.9. Quantitative photolysis and product analysis for (8)

Separate solutions of **8** (0.47 mM in 25 mM Na phosphate, pH 7.0 containing 5 mM dithiothreitol) were irradiated for varying times (20 or 25 s) in 1 mm path length cells (Rayonet Photochemical Reactor). The solutions were analysed by anion-exchange HPLC (mobile phase as in Section 4.8) and the extent of photolysis of each solution was determined by comparison of peak heights with those of non-irradiated controls. Aliquots of the photolysed solutions were also subjected to quantitative amino acid analysis. Measured glycine concentrations were 68–69% of the expected values from the extent of photolysis and were not affected by the concentration

of dithiothreitol. The stock solution (20.9 mM) was also subjected to quantitative amino acid analysis and the measured free glycine contamination was 0.35%.

In a further experiments, separate solutions of **8** and **15** (0.51 mM) in 25 mM Na phosphate, pH 7.0 were irradiated for increasing times (0–90 s) in a 1-mm path length cell and the extent of photolysis was monitored after each irradiation interval by UV–vis spectroscopy.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.05.045. These data include MOL files and InChiKeys of the most important compounds described in this article.

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