Synthesis and Photophysical Characterization of a New, Highly Hydrophilic Caging Group

Klaus Schaper,*[a] S. Abdollah Madani Mobarekeh,[a][‡] and Christof Grewer[b]

Dedicated to Prof. Dr. L. Birkofer on the occasion of his 90th birthday

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o-Nitrobenzyl-protected bioactive compounds are useful tools in biophysics, allowing controlled photorelease of biologically active compounds with high temporal and spatial precision. Thus, it is possible to study biological processes, such as neurotransmitter-receptor interaction, and many other processes, in much more detail than before. In this respect, these caged compounds have become established as extremely useful tools. In some cases, however, their biological properties (the caged compound should not interact with the biological system), their photochemical properties (quantum yield and kinetics of the photorelease), and their physical properties (high hydrophilicity) are not satisfactory. In order to address the last problem, we examined means to increase the hydrophilicity of caged compounds based on the *o*-nitrobenzyl moiety. Here, we report the synthesis and the photochemical and biological characterization of a new caged D-aspartate derivative with vastly improved hydrophilicity, compared to derivatives reported previously, and satisfactory photophysical properties. Caged compounds with this improved *o*-nitrobenzyl group may thus represent a valuable new tool for different kinds of biophysical investigations.

Introduction

The photoreactivity of *o*-nitrobenzaldehyde, namely the light-induced formation of *o*-nitrosobenzaldehyde, was discovered as early as 1901, by Ciamician and Silber.[1] On the basis of this discovery, Baltrop[2] and Woodward[3] introduced the *o*-nitrobenzyl moiety as a photolabile protecting group. Only in recent years, though, has this method become widely used in organic synthesis.[4−6] Very recently, *o*-nitrobenzyl groups were introduced as photolabile linkers in solid-phase synthesis[7,8] and as photoswitches to release guest molecules from hemicarceplexes.[9]

The synthesis of bioactive compounds with biological activity controllable through a photolabile protecting group began in the seventies. These compounds, which were named caged compounds, were introduced by Engels[10,11] and Kaplan[12] and have proven to be useful tools in many biophysical studies. Through the use of laser technology, the bioactive compounds can be released from the caged compounds in microseconds either within cells[13−15] or on cell surfaces.[16−24] The *o*-nitrobenzyl group has proven useful for the photodeprotection of various functional groups: namely carboxylates,[21−23,48] phosphates,[12,25] amines,[26−28] amides,[29,30] carbamates,[16] phenols,[18] alcohols,[31] and cysteine and methionine thiol groups within proteins,[32,33] as well as inorganic ions (e.g. calcium),[34] nitrogen oxide,[35] and protons.[36] However, the caged compounds do not always have the desired properties, such as (i) satisfactory water solubility and high hydrophilicity, (ii) stability against thermal hydrolysis, (iii) high excitation wavelength in order to avoid cell damage ($\lambda_{max} > 320$ nm), (iv) high quantum yields, (v) fast photoreaction (within a few μs), (vi) inertness with respect to the biological system under investigation and any process within the cell, and (vii) biological inertness of the side products of the photoreaction. It is not yet possible to predict the behavior of the caged compounds in biological systems; they in some cases show some residual activity or act as inhibitors.[21] On the other hand, their physical (i) and chemical (ii) properties can easily be predicted and the photochemical (iii to v) properties of the compounds can be calculated by theoretical methods.[37]

At present, the most widely used caging group is the *o*-nitrobenzyl moiety.[38,39] The introduction of an α-carboxy

[a] Institut für Organische Chemie und Makromolekulare Chemie, Lehrstuhl I, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany
Fax: (internat.) + 49-(0)211/81-14324
E-mail: schaper@klaus-schaper.de
[b] Max-Planck-Insitut für Biophysik, Kennedyallee 70, 60596 Frankfurt, Germany
Fax: (internat.) +49 (0)69/6303 305
E-mail: grewer@mpibp-frankfurt.mpg.de
[‡] Some of these results will be part of the PhD thesis of S. A. M. M., Heinrich-Heine-Universität, Düsseldorf.
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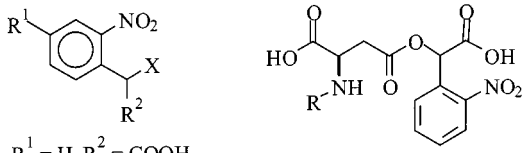


1a $R^1 = H, R^2 = COOH$
1b $R^1 = COOH, R^2 = COOH$
1c $R^1 = H, R^2 = H$

2a R = Me
2b R = H

Scheme 1

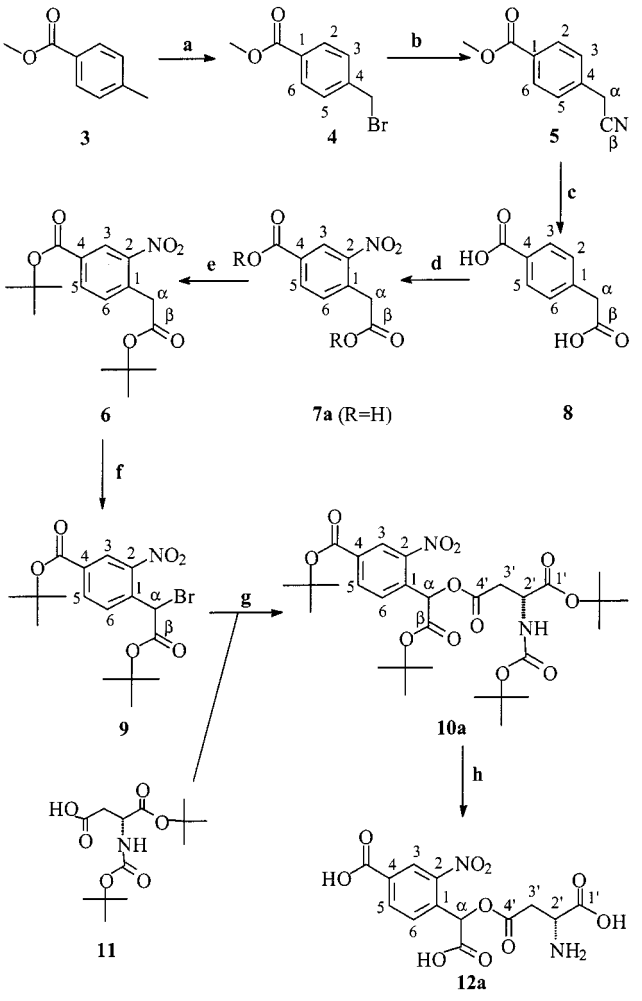
group into the *o*-nitrobenzyl system, thus creating the α-carboxy-2-nitrobenzyl (α-CNB) system **1a** (Scheme 1), made it possible to improve the water solubility, the hydrophilicity, and the photophysical properties (iv and v) of the caged compound.[16,19,20,23] However, the nitrobenzyl moiety of the α-CNB caging group is rather hydrophobic. In some cases this may cause problems, due to possible association of this moiety with hydrophobic sections of proteins. To prevent such possible hydrophobic interactions, we planned to increase the hydrophilicity of the α-CNB group through the introduction of an additional carboxylate, thus creating the new α,4-dicarboxy-2-nitrobenzyl (α,4-DCNB) protecting group **1b**. The introduction of this carboxylate should result in better distribution of the compound between the aqueous phase and the hydrophobic phase, with the compound more likely to be found in the aqueous phase. The additional carboxylate function might also increase the water solubility of **1b** compared to that of **1a**, which would be an additional advantage of this new photolabile protecting group, especially for protection of substrates with poor water solubility. Here we show the development of this caging group and prove its utility with a new caged D-aspartate protected as the β-ester with this group.

Results and Discussion

Synthesis

The synthesis of a β-caged α-CNB *N*-methyl-D-aspartate (NMDA) **2a** (Scheme 1) was published previously.[21] Starting from *N*-BOC-NMDA (BOC: *tert*-butyloxycarbonyl), both carboxy groups were esterified with *tert*-butyl α-bromo-2-(nitrophenyl)acetate (**1a**, X = Br), and the more reactive ester (in the position α to the methylamino group) was subsequently cleaved under basic conditions. The caged NMDA **2a** was obtained from this intermediate by removal of the BOC and *tert*-butyl protecting groups with trifluoroacetic acid (TFA). A similar approach to the synthesis of a β-caged α-CNB D-aspartate **2b** was not successful. The selective deprotection of the more reactive ester was not applicable to the corresponding α-CNB D-aspartate diester.

We therefore developed a modified procedure that used an α-*tert*-butyl ester of *N*-BOC-D-aspartate **11** (Scheme 2) and *tert*-butyl α-bromo(2-nitrophenyl)acetate (**1a**) (X = Br). Afterwards, the *N*-BOC group and the *tert*-butyl esters were cleaved with TFA to produce the desired β-caged α-CNB D-aspartate **2b**.[40] By this procedure, we synthesized a new caged D-aspartate derivative incorporating the photolabile α,4-DCNB protecting group (Scheme 2).

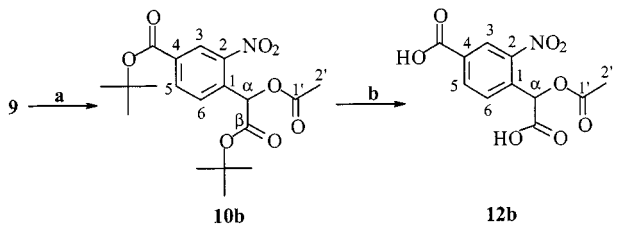


Scheme 2. Synthesis of β-(α,4-DCNB)- D-aspartate **12a**. a: Br_2, benzene, *hν*, 86 %, b: KCN, EtOH, H_2O, 74 %, c: H_2SO_4, H_2O, 87 %, d: HNO_3, H_2SO_4, 70 %, e: isobutene, H_2SO_4, 20 °C, $CHCl_3$, 57 %, f: NBS, CCl_4, 34 %, g: DBU, benzene 58 %, h: TFA, toluene, 88 %

Starting from *p*-toluic acid methyl ester **3**, we obtained (*p*-carboxyphenyl)acetic acid **8** in three steps by standard methods.[41–43] Selective nitration in the 3-position yielded the dicarboxylic acid **7a**.[44] Because of the highly hygroscopic nature of this compound, it was not possible to obtain an elemental analysis, and so for that purpose we converted **7a** into its dimethyl ester **7b** by standard methods.[45] Double esterification of the dicarboxylic acid **7a** with isobutene under standard conditions[46] gave only minute conversion efficiencies. We therefore warmed the solution to room temperature in an autoclave and stirred for two weeks. Finally, bromination yielded the desired reagent **9**.

Coupling of reagent **9** with an α-carboxy- and amino-protected D-aspartate **11** provided the tetra-*tert*-butyl-protected precursor of the β-caged α,4-DCNB D-aspartate **10a**. Deprotection with TFA yielded the final product **12a**, again as a mixture of diastereoisomers. These coupling and deprotection steps were optimized with acetate as a model sys-

tem for D-aspartate prior to the final synthesis, to yield the model compounds **10b** and **12b** (Scheme 3).



Scheme 3. Synthesis of the model compound DCNB-acetate **12b**. **a**: DBU, THF, HOAc 63 %, **b**: TFA, toluene, 59 %

Most synthetic steps were optimized, and the yields are therefore generally high (70% to 90%). However, the synthesis of compounds **6** and **9** produced relatively poor yields (57% and 34%, respectively). Nonetheless, most of the unchanged starting material in these reaction steps could be recovered, and the yields in terms of consumption of starting material were reasonable or better (100% and 68%, respectively). Only the key step of the reaction, the coupling of the photolabile protecting group to the doubly protected D-aspartate, still produced unsatisfactory yields (58%).

Nevertheless, the total yield of this eight-step synthesis was, at 9%, quite satisfactory. In most biophysical studies, only a few microliters of a micromolar to millimolar solution are needed for each experiment. Therefore even though the yields in some steps are still not satisfying, it is no problem to obtain enough compound for thorough biophysical studies.

The synthetic method described above should not only be useful for the synthesis of β-caged α,4-DCNB D-aspartate **10a**, but also be easily applicable to other carboxylic acids. α-CNB-protected derivatives of aspartate, NMDA, kainate, glutamate, and others have been prepared by very similar procedures, and so one might expect that it would be feasible to use the α,4-DCNB group as a protecting group for carboxylic acids other than those in D-aspartate. Some of these compounds are under investigation. With slight modifications in the procedure, the α-CNB group can be attached to other functional groups than the carboxylic moiety, such as phosphates, carbamates, phenols, thiols, and some others. There is no apparent reason why the bromide **9** described above should not be used in such a way, and this reagent may therefore become a generally used, improved building block in the synthesis of caged compounds.

All of these compounds should profit from the increased hydrophilicity. From observed behavior in the preparation of its aqueous solutions, it appears that the water solubility of the β-caged α,4-DCNB D-aspartate **10a** is better than that of the α-CNB derivative **2b**. This might become important in the preparation of caged compounds in which the leaving group is not as water-soluble as it is in this case.

Photochemical Characterization

Kinetic Properties

The transient absorption at 430 nm produced by **12a** upon irradiation at 308 nm (pH 7.1) is shown in Figure 1. This absorption is caused by transient formation of the *aci*-nitro intermediate **15** (Scheme 4), with an absorption maximum at about 430 nm (see below). It decays with double-exponential kinetics with the following parameters: A_0 = 0.03, A_1 = 0.66, k_1 = 9050±58 s^{-1}, A_2 = 0.31, k_2 = 1970±500 s^{-1}, total amplitude normalized to 1. This behavior is typical of the photochemistry of *o*-nitrobenzyl compounds.[21,22,23,40,47,48] According to earlier proposals[49,50,51,52] and our newer theoretical investigations, the existence of the two kinetic components can most plausibly be attributed to the possible stereoisomerism of the *aci*-nitro intermediate, which can be formed as the (*Z*) isomer **15**^{Z} or the (*E*) isomer **15**^{E}.[40] The observed rate constant of the fast phase is about a quarter of the rate constant for the respective reaction of **2b** [k_1 = (3.85±0.40)·10^4 s^{-1}]. The slow and minor phases of the reaction decay with about the same rate (for **2b**, k_2 = 2400±310 s^{-1}) for both compounds. Therefore, the improved water solubility is linked somehow to a slower photorelease of the active compound.

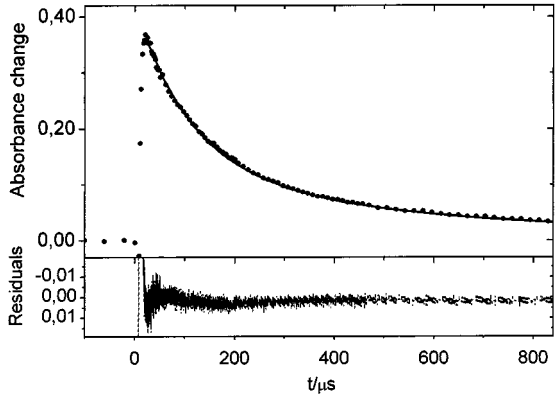
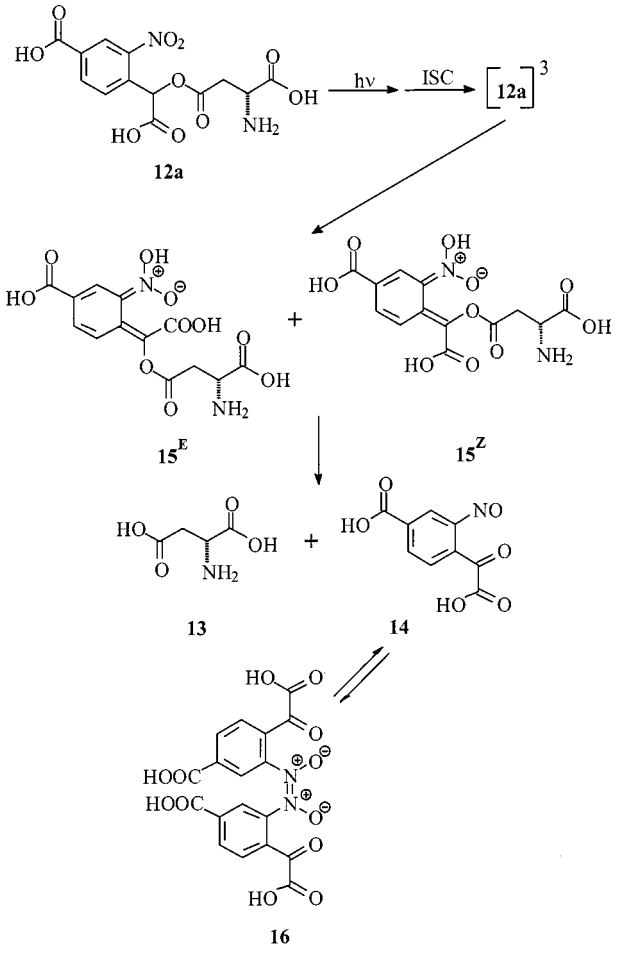


Figure 1. The transient absorption upon irradiation at t = 0 s of 1 mM β-caged α,4-DCNB aspartate at pH = 7 (100 mM phosphate buffer); the sample was irradiated with a 10-ns laser flash at 308 nm and the absorption was measured at 430 nm; the solid line presents the best fit of the data (A_0 = 0.013, A_1 = 0.27, t_1 = 105 μs, A_2 = 0.13, t_2 = 42 μs), the filled circles represent the measured data (1 out of 50 data points is shown), the lower panel shows the residuals of the fit

The transient absorption measurements were repeated at several wavelengths between 350 nm and 620 nm (data not shown). From these data the absorption spectrum of the intermediate **15** at several intervals after the laser flash was generated (Figure 2); the absorption maximum of this intermediate was about 460 nm. In comparison with the typical absorption of *aci*-nitro intermediates[21−23,40,47,48] seen with the α-CNB protecting group, the absorption was red-shifted by about 30 nm. If the *aci*-nitro intermediate is viewed as a cyanine dye analogue chromophore, this behavior is in agreement with Dewar's[53−55] rule. Such a description can be justified by the shapes of the HOMO and LUMO (Figure S1 in the Supporting Information; see also footnote on



Scheme 4. Proposed mechanism of photolysis of β-DCNB-D-aspartate

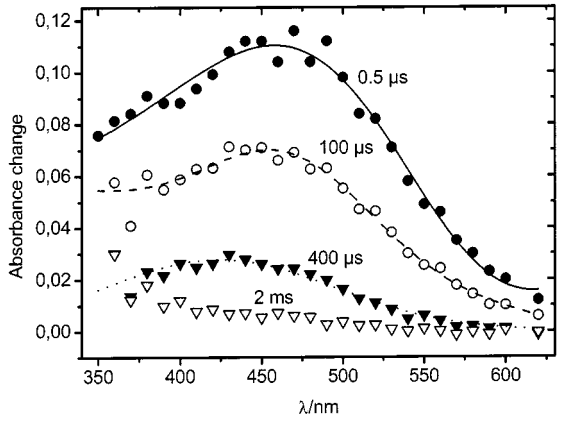


Figure 2. UV/Visible spectra of the transient absorption recorded 0.5 µs, 100 µs, 400 µs, and 2 ms after the laser flash; the spectra were constructed from several transient spectra, recorded at wavelengths of 350 to 630 nm (stepwidth 10 nm)

the first page of this article) obtained from ab initio calculations (see below).

Irradiation of a sample of **12a** with 100 consecutive laser pulses resulted in complete photolysis; the typical spectral change is shown in Figure 3. The characteristic absorption at 320 nm[56] is caused by the formation of the azodioxide[57] **16**, the dimer of the nitroso compound. The typical absorption of the nitroso-monomer around 750 nm had a very low extinction coefficient and could not be observed. The small but distinct absorption of **16** at 430 nm caused the observed offset (A_0) in the transient absorption spectra (Figure 1).

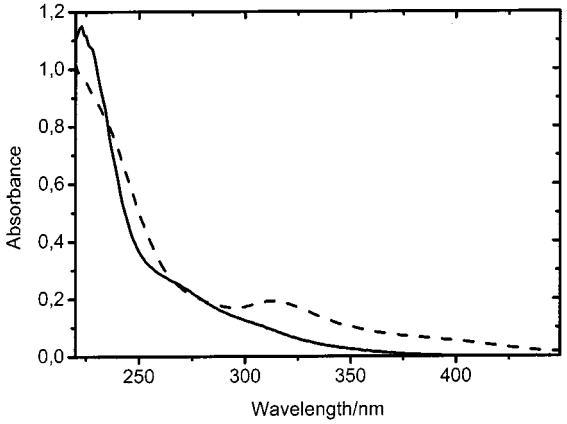


Figure 3. UV/Visible spectrum of 12a (50 µM, 100 mM phosphate buffer. pH 7.1) before photolysis (straight line) and of the reaction products (dashed line) after complete photolysis (100 laser pulses of 15 mJ, 330 µL solution)

pH-Dependence

The decay kinetics of the *aci*-nitro intermediate were measured at different pH values. Both rate constants demonstrated a relatively weak, but significant pH-dependence, as shown in Figure 4. The data are summarized in Table 1. As reported before for compounds with the α-carboxynitrobenzyl protecting group **1a**,[22,23] both rate constants increased with decreasing pH in the range between pH = 10 and pH = 5. A decrease in the rate constant below pH = 5 has been reported for caged compounds with an α-carboxy group.[21,22] For the smaller of the two rate constants we found this decrease already at pH = 5, as described earlier for **2b**.[40] This behavior was in agreement with new theoretical investigations, which attributed the increased rate at lower pH to the protonation of the *aci*-nitro group and the

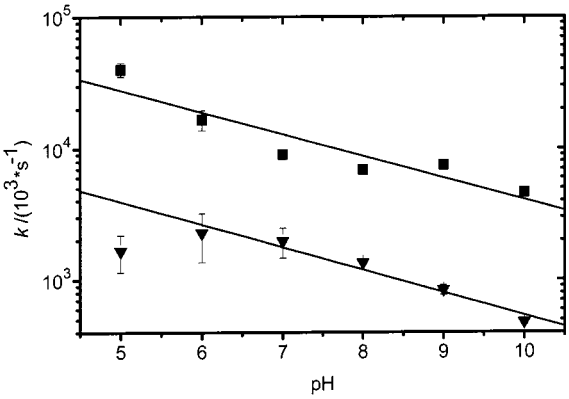


Figure 4. pH-Dependence of the rate constants of the fast (k_1) and the slow (k_2) exponential components of the decay of the *aci*-nitro intermediates **15**

Table 1. The rate constants for the decay of the *aci*-nitro intermediate formed from the β-DCNB glutamate **12a** upon irradiation with a laser at 308 nm at different pH values; the quantum yield for the consumption of starting material **12a** is also shown; the buffer solutions were acetate (pH = 5.0), phosphate (pH = 6.0, 7.0, 8.0), and borate (9.0, 10.0) at 100 mM; the temperature was 22 °C; the data were obtained by transient UV/Vis spectroscopy at 430 nm

pH	$k_1/(10^3 s^{-1})$	$k_2/(10^3 s^{-1})$	Φ
5	41.1±4.9	1.67±0.52	0.056
6	16.7±2.9	2.28±0.91	0.13
7	9.05±0.05	1.97±0.50	0.14
8	6.89±0.38	1.35±0.06	0.070
9	7.49±0.30	8.37±0.07	0.062
10	4.63±0.33	4.77±0.02	0.030

decreased rate at pH < 5 to protonation of the α-carboxy group.[37] It thus seems that (i) the (*E*) and (*Z*) isomers have different α-carboxy group pK_a values and (ii) the additional carboxy group in the 4-position has only a small influence on the pH-dependence of the rate constants.

Typical bimolecular rate constants for protonation reactions of acids in aqueous solution are in the range of 10^{10} $L \cdot mol^{-1} \cdot s^{-1}$ to 10^{12} $L \cdot mol^{-1} \cdot s^{-1}$.[58] From this data one can estimate a pseudo-monomolecular rate constant of 10^3 s^{-1} to 10^5 s^{-1} at pH = 7. Therefore, the protonation of the *aci*-nitro compound could well be rate-limiting (the rate constants for *aci*-nitro decay at pH = 7 are 9050 s^{-1} and 1970 s^{-1}; see above). In this case, one would expect a general acid catalysis mechanism. However, we have not yet measured the rate constant for the decay of the *aci*-nitro intermediate as a function of buffer concentration at constant pH in order to verify this assumption. In case of acid catalysis, a tenfold acceleration per pH unit would be expected. Here we observed only a 1.2- to 1.5-fold acceleration, in agreement with previous reports on α-CNB-caged and nitrobenzyl-caged carboxylates,[19] carbamates,[16] and amides.[59] It is therefore likely that the decay rate of the *aci*-nitro intermediate is dominated by a pH-independent reaction for these compounds.

Quantum Yield

The quantum yield of the photoreaction was measured with a previously reported method,[19–24,40] by plotting the transient absorption of the *aci*-nitro intermediate after consecutive laser shots as a function of the number of laser pulses (Figure 5). The quantum yield at pH 7 was 0.14±0.01, about 25% lower than the quantum yield of **2b**. From these measurements the extinction coefficient of the intermediate was determined to ε = 3000 $M^{-1} \cdot cm^{-1}$ at 440 nm and pH 7.0, with an estimated error of 1000 $M^{-1} \cdot cm^{-1}$ (see Exp. Sect.).

Measurements between pH 5 and pH 10 revealed a strong interdependence between quantum yield and pH, with a maximum between pH 6 and 7 (Figure 6 and Table 1). This behavior is in good agreement with recently published data for compound **2b**.[40] It is reasonable to assume that this behavior can be attributed to the protonation of the α-carboxy group in the same manner as for the pH-dependence of the rate constant. In this case we already find a distinct change of behavior around pH = 5. Obviously, the α-carboxy group in **12a** has a higher pK_a than the one in the *aci*-nitro intermediate, which is in agreement with what could be expected from the resonance structures. Again, the 4-carboxyl group seemed to have no influence on the pH-dependence. This can be explained by the low pK_a value for this carboxyl group, which can be estimated as pK_a = 3.5 (water, 28.5 °C) by comparison with *m*-nitrobenzoic acid[60] as reference compound.

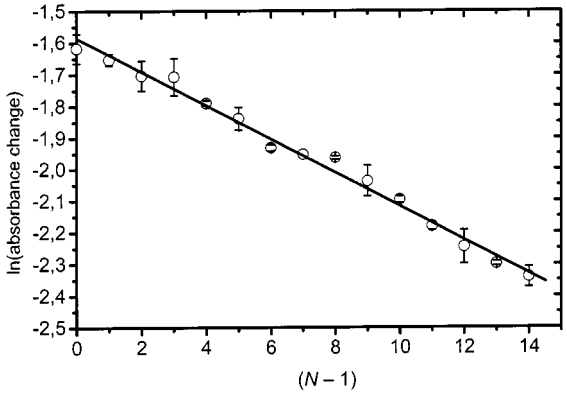


Figure 5. A sample of α,4-DCNB-aspartate **12a** (500 μM, 100 mM phosphate buffer, pH = 7.0, 50 μL) was irradiated with consecutive laser pulses (10 ns, 308 nm, 10 mJ); the logarithm of the transient absorption immediately after the laser pulse (0.5 μs) was plotted versus the number of the laser pulses −1; from the slope of this linear fit we obtained the quantum yield and from the intercept we were able to estimate the extinction coefficient of the aci-nitro intermediate; each data point represents the mean of three experiments

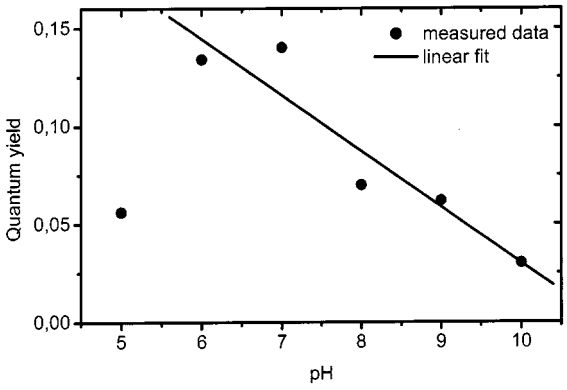
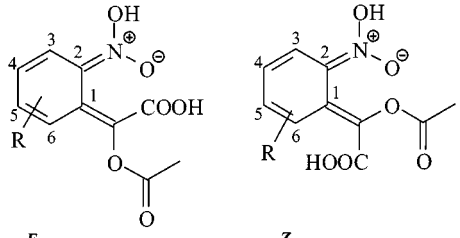


Figure 6. The quantum yield for the photorelease of aspartate from α,4-DCNB-aspartate **12a** (500 μM) is shown (filled circles); a linear fit (straight line) for pH ≥ 6 is shown; below pH = 6 the quantum yield becomes smaller again

Ab initio Calculations

In order to understand the shifts in the spectra and in the photodissociation rate constants introduced by the ring carboxy substituent theoretically, we performed ab initio calculations. Calculations on the STO6-31G**[61–67] level

were carried out for model compounds **17**^{E}, **17**^{Z} (as model systems for the *aci*-nitro intermediates of **2b**), **18**^{E}, **18**^{Z} (as model systems for the *aci*-nitro intermediates of **12a**), and **19**^{E}, **19**^{Z} (Scheme 5). We assumed, from earlier investigations,[37] that the reactive species at pH = 7 was the one in which the *aci*-nitro group was protonated and the carboxylates were deprotonated.



17^{E}: R = H **17**^{Z}: R = H
18^{E}: R = 4-COOH **18**^{Z}: R = 4-COOH
19^{E}: R = 5-COOH **19**^{Z}: R = 5-COOH

Scheme 5. Model compounds used in our ab initio studies

From the orbital coefficients in the HOMO and LUMO, one can understand the observed bathochromic shift, caused by the electron-withdrawing carboxylate function (Figure S1, Supporting Information; see also footnote on the first page of this article).

Earlier, we had developed a method to predict the relative reactivity of different *aci*-nitro intermediates of **1c** depending on their state of protonation,[37] based on Kloopmans[68] Equation (1), where ΔE_{Tot} is the total energy, q_N and q_E are the charges of the nucleophilic and the electrophilic centers, respectively, R is the distance from the nucleophilic to the electrophilic center, c_{HOMO} and c_{LUMO} are the coefficients of the HOMO at the nucleophilic center and of the LUMO at the electrophilic center, E_{HOMO} and E_{LUMO} are the corresponding orbital energies, and β_{HL} is the resonance integral.

$$\Delta E_{Tot} = \frac{1}{4\pi\varepsilon_0}\frac{q_N q_E}{R_{NE}} + \frac{2(c_{HOMO}c_{LUMO}\beta_{HL})^2}{E_{HOMO} - E_{LUMO}} \quad (1)$$

In this model we described the nucleophilic attack of the *aci*-nitro oxygen at the benzylic position. We were able to show that the intermediates were especially reactive when the *aci*-nitro oxygen was protonated. All the necessary data to calculate ΔE_{Tot} according to Equation (1) can be obtained from semiempirical or ab initio calculations, except for $\beta_{\mathrm{H\Lambda}}$. Our results were independent of β_{HL}. Here, we applied this method to the relative reactivities of **17** to **19**. In order to obtain an estimate of β_{HL}, we used an approximation by Pariser and Parr [Equation (2)],[69] where β_{HL} is the resonance integral and R is the distance between the two atoms.

$$\beta_{HL}(R) = -6442eV * \exp\left(\frac{-5.6864R_{NE}}{\text{Å}}\right) \quad (2)$$

We were thus able to obtain approximations for the Coulomb interaction [first term of Equation (1)] and for the orbital interaction [second term of Equation (1)]. The data are summarized in Table 2. From these data we concluded that the orbital interaction was negligible in comparison with the Coulomb interaction. This was not surprising, since the Coulomb interaction scales with 1/*r*, while the orbital interaction scales with an exponential decay function. The introduction of a carboxy group into the 4-position of **17** resulted in a reduction of the Coulomb interaction in the (*E*) and (*Z*) isomers. Thus, one could expect a reduction in the rate constants for the *aci*-nitro decay of both components of the double exponential decay, in good agreement with the experimental results (see above). From a simple analysis of the resonance structures of **18** one might expect that the electron-withdrawing group would reduce the electron density at the *aci*-nitro oxygen compared to that in **17**, but the ab initio calculations showed that matters were more complicated. The electron density was altered along the whole π-system, and as an effect the electron density at the *aci*-nitro oxygen was increased. At the same time the electron density at the benzylic position was also increased, and the reduction in the positive charge in this position was the reason for the reduced Coulomb interaction. If a carboxy group was attached to **17** in the 5-position (compound **19**) instead of in the 4-position (compound **18**), our calculations showed a reduced Coulomb interaction as well. This effect was much smaller, however, and for the *aci*-nitro decay of **19** one would expect rates intermediate between those observed for **17** and **18**. We therefore plan the synthesis of compounds of type **19** to test this hypothesis.

Semiempirical calculations on the AM1[70,71] level provided similar results for **17** and **18**. However, the results for **19** were different from those obtained from ab initio methods (see Table S1 in the Supporting Information). Obviously, semiempirical calculations do not provide enough accuracy.

Biological Characterization of Photorelease

The utility of the new β-caged α,4-DCNB D-aspartic acid was tested by using current recordings from human embryonic kidney cells expressing the excitatory amino acid carrier 1 (EAAC1). Firstly, we tested whether β-caged α,4-DCNB-D-aspartate **12a** inhibited EAAC1. A solution of free D-aspartate **13** (100 μM) was applied to the cell with a fast solution-exchange device. Afterwards we repeated the experiment with the same cell and coapplied either 1 mM of α-CNB-D-aspartate **2b** or β-caged α,4-DCNB-D-aspartate **12a** together with 100 μM of free D-aspartate **13**. The current response of the cell in all three experiments was nearly the same (Figure 7). When the caged compound was applied to the cell in the absence of aspartate, a small, but significant activation of the EAAC1 current was observed;

Table 2. Some results of ab initio calculations at the STO 6-31 G** level for the model *aci*-nitro intermediates **17** to **19** in the (*E*) and (*Z*) configuration; the resonance integral β_{HL}, which describes the interaction of HOMO and LUMO at the point of the nucleophilic attack, is estimated by a simple distance-dependent equation [Equation (2)] from the distance of these centers; from these results the orbital and the Coulomb interaction of these two centers is calculated according to Kloopman's equation [Equation (1)]

Compound	17^E	18^E	19^E	17^Z	18^Z	19^Z
E_{LUMO}/eV	4.88	7.62	7.80	4.79	7.62	7.66
E_{HOMO}/eV	−4.31	−1.53	−1.48	−4.37	−1.53	−1.50
ΔE/eV	9.19	9.15	9.28	9.16	9.15	9.16
c_{HOMO}	0.23652	0.34487	0.32030	0.34223	0.30771	0.28435
c_{LUMO}	0.51223	0.42746	0.44225	0.46533	0.47239	0.49461
R_{NE}/A	2.996	2.976	3.007	3.008	3.011	3.021
$\beta_{HL}/(10^{-4}$ eV)	−2.57	−2.88	−2.42	−2.40	−6.80	−2.23
q_N	−0.643026	−0.672505	−0.675361	−0.66417	−0.694615	−0.694504
q_E	0.302936	0.253696	0.265325	0.30724	0.263785	0.287544
$\Delta E_{orbital}/(10^{-5}$ J/mol)	−2.0	−3.8	−2.4	−3.1	−2.5	−2.1
$\Delta E_{Coulomb}/(10^3$ J/mol)	−90.31	−79.6	−82.8	−94.2	−84.5	−91.82
Total energy/Hartree	−886.851	−1073.834	−1073.832	−886.862	−1073.845	−1073.835

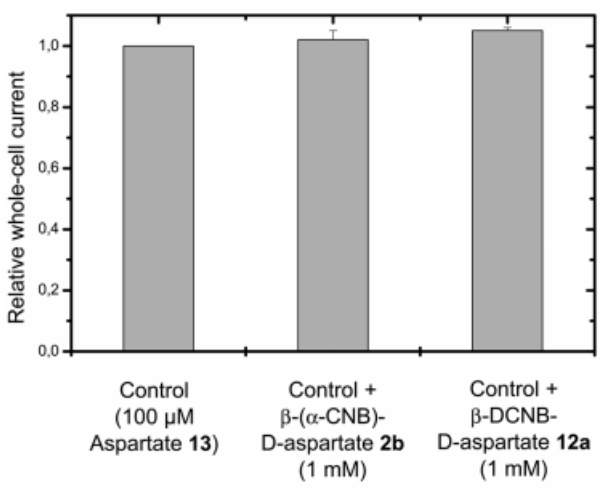


Figure 7. Whole-cell current recording for EAAC1 expressed in HEK293 cells with 100 µm free aspartate **13** (column 1), 100 µM free D-aspartate **13** in the presence of 1 mM β-(α-CNB)-D-aspartate **2b** (column 2), and 100 µM free D-aspartate **13** in the presence of 1 mM β-(α,4-DCNB)-D-aspartate **12a** (column 3)

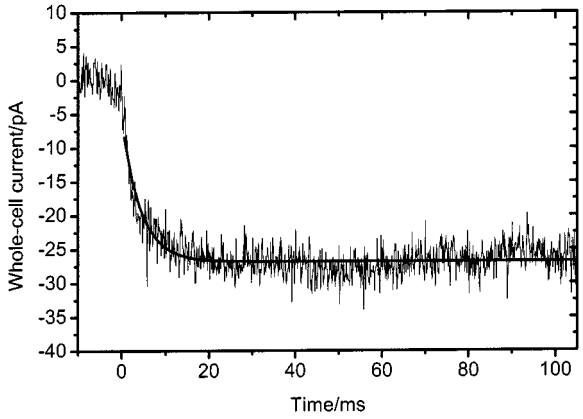


Figure 8. Laser-pulse photolysis experiment with 150 µM β-(α,4-DCNB)-D-aspartate **12a**; aspartate was released upon application of a 345 nm, 10 ns laser pulse (400 mJ/cm^2); the current was recorded at $U = 0$ mV; the liberated D-aspartate concentration was estimated as 15 µM

this may have been due to slight contamination of **12a** with free aspartate or to residual activity in the caged compounds **12a** and **2b**. If the compounds have no residual activity, the levels of contamination can be estimated from these experiments as 0.2% and 0.5%, respectively. The experiments showed that both α-CNB-D-aspartate **2b** and β-caged α,4-DCNB D-aspartate **12a** should be suitable tools for the investigation of EAAC1, as neither affected EAAC1 function before photolysis.

In a second experiment we applied 150 µM β-caged α,4-DCNB D-aspartate **12a** to the cell with the same rapid solution-exchange device. Afterwards, β-caged α,4-DCNB D-aspartate **12a** was photolyzed to release free D-aspartate **13**. Upon release, the typical response to a free D-aspartate concentration jump was observed. The current rose with a time constant of 4.3 ms to a new steady-state level. As demonstrated previously,[40] and in contrast to results obtained by application of glutamate concentration jumps, D-aspartate did not evoke substantial transient currents in EAAC1. (Figure 8). Rise times as fast as those observed here cannot easily be resolved by flow methods, but have previously been determined with other caged D-aspartate derivatives **2b**.[40]

Conclusion

The new β-caged α,4-DCNB D-aspartate **12a** should have a vastly increased hydrophilicity in comparison with the known derivative **2b**.[40] Therefore, the new β-caged α,4-DCNB D-aspartate **12a** is a useful tool for biophysical investigations when other caging groups such as α-CNB do not provide sufficient hydrophilicity. Furthermore, the photolysis quantum yield is sufficiently high and the photorelease of the active compound is fast enough for most purposes, although slightly slower than photorelease of carboxylic acids from the α-CNB precursor. Therefore, com-

pounds with the α,4-DCNB protecting group will be useful tools if increased hydrophilicity is needed in the caged compound. On the other hand, it has to be taken into account that the synthesis of the α,4-DCNB-protecting group is much more costly in terms of the number of reaction steps than the synthesis of the α-CNB protecting group, or even more so with regard to the simple nitrobenzyl group (six, two, and one reaction step(s), respectively). The introduction of polar substituents, as shown here, may also be useful if the apolar caging groups interact with hydrophobic regions of the protein under investigation, possibly resulting in its inhibition. Similar approaches for decreasing the hydrophobicity may be also applicable to caging groups other than the nitrobenzyl system.

We were able to understand the slower release of D-aspartate from β-caged α,4-DCNB D-aspartate **12a** relative to its release from α-CNB D-aspartate **2b**[40] by a simple model based on our earlier work[37] and on ab initio calculations. This work further suggests that a compound **19** with an additional carboxy group in the 5-position of the α-CNB group **17**, rather than in the 4-position (α,4-DCNB, **18**), would release the active compound more rapidly than the α,4-DCNB protected compounds would. Further investigations will have to prove this hypothesis.

It was furthermore demonstrated here that the new caging group, when used to protect D-aspartic acid, produces a caged compound that is inactive with respect to the glutamate transporter subtype EAAC1. Laser-pulse photolysis, however, generates D-aspartic acid and permits time-resolved measurement of EAAC1 associated currents, thus showing the usefulness of the new compound for biological applications.

Experimental Section

Syntheses

General Remarks: The BOC-α-(*tert*-butyl)-D-aspartate (**11**) was obtained from Bachem Biochemica GmBH. For column chromatography, either 60 silica gel (0.063−0.200 mm) from Merck or Sephadex LH20 from Pharmacia were used. 200 MHz ^{1}H NMR spectra were measured on a Bruker DXR 200, 300 MHz ^{1}H NMR and 75 MHz ^{13}C NMR were measured on a Varian VXR 300, and 500 MHz ^{1}H NMR and 125 MHz ^{13}C NMR were measured on a Bruker DXR 500. IR spectra were recorded on a Bruker Vector 22, UV spectra were recorded on a Perkin Elmar Lambda 19. Melting points were determined with a Reichardt Thermovar apparatus, Büchi 510. The ^{13}C NMR spectra were assigned by increment systems[72] and by comparison with reference spectra.[73] ^{1}H NMR spectra were assigned by increments.[72] IR spectra were assigned by using reference data.[74]

The synthesis of the compounds **4**, **5**, **6**, **7a**, **7b**, **8**, **9**, **10b**, and **12b** and their spectroscopic data are described in the Supporting Information (see footnote on the first page of this article).

4-*O*-[α,4-Bis(*tert*-butoxycarbonyl)-2-nitrobenzyl] 1-*O*-*tert*-Butyl (*R*)-*N*-(*tert*-Butoxycarbonyl)aspartate (10a): A mixture of 1-*O*-(*tert*-butyl) (*R*)-*N*-(*tert*-butoxycarbonyl)aspartate (**11**, 150 mg, 0.520 mmol), *tert*-butyl α-bromo-α-(4-*tert*-butoxycarbonyl-2-nitrophenyl)acetate (**9**, 250 mg 0.600 mmol), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 100 mg, 0.650 mmol) in absolute benzene (15 mL) was heated under reflux for 5 h. The mixture was allowed to cool to room temp., and water (20 mL) and ethyl acetate (20 mL) were then added. The phases were separated, and the aqueous layer was extracted with ethyl acetate (2 × 10 mL). The combined organic layers were dried with sodium sulfate and concentrated under reduced pressure. The product **10a** was purified by column chromatography (silica gel, *n*-hexane/ethyl acetate, 4:1) to yield a roughly 1.3:1 mixture of diastereomers (estimated from the ^{1}H NMR integrals of the benzylic proton) as a pale brown viscous oil (190 mg, 0.300 mmol, 58%). ^{1}H NMR ($CDCl_3$ 300 MHz): δ = 8.56 and 8.57 (2 d, $^4J_{H,H}$ = 1.4, $^4J_{H,H}$ = 1.4 Hz, 1 H, Ar-3-H), 8.23 and 8.22 (2 dd, $^3J_{H,H}$ = 8.2, $^3J_{H,H}$ = 8.2, $^4J_{H,H}$ = 1.8 and 1.8 Hz, 1 H, Ar-5-H), 7.72 (d, $^3J_{H,H}$ = 8.2 Hz, 1 H, Ar-6-H), 6.85 and 6.83 (2s (signal integral: 1.3:1), 1 H, α-H), 5.57 and 5.52 (2 d, $^3J_{H,H}$ = 8.4, $^3J_{H,H}$ = 7.5 Hz, 1 H, N-H), 4.55−4.45 (m, 1 H, 2′-CH), 3.30−2.90 (m, 2 H, 3′-H), 1.67−1.62 [s, 9 H, Ar−CO−C(CH$_3$)$_3$], 1.45 and 1.44 [2 s, 9 H, N−CO−C(CH$_3$)$_3$], 1.40, 1.39, 1.38 [3 s, 18 H, Alk−CO−C(CH$_3$)$_3$]. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 169.59 and 169.38 (4′-C, 1′-C), 165.53 and 165.45 (β-C), 163.02 (Ar-COO-*t*Bu), 155.48 and 155.41 (NH−CO−O), 148.09 and 148.03 (Ar-2-C), 133.72 (Ar-5-C), 133.201 and 132.935 (Ar-1-C and Ar-4-C), 129.05 and 128.98 (Ar-6-C), 125.90 (Ar-3-C), 84.12 (Ar−CO−O*C*(CH$_3$)$_3$], 82.85 and 82.61 and 82.58 [β-C−O*C*(CH$_3$)$_3$ and 1′-C−O*C*(CH$_3$)$_3$], 79.93 [NH−CO−O*C*(CH$_3$)$_3$], 70.35 and 70.23 (α-C), 50.43 and 50.31 (3′-C), 36.78 and 36.73 (2′-C), 28.31 [NH−CO−OC(*C*H$_3$)$_3$], 28.09 [Ar−CO−OC(*C*H$_3$)$_3$], 27.80 and 27.71 and 27.68 [(β-C−OC(*C*H$_3$)$_3$ and 1′-C−OC(*C*H$_3$)$_3$]. The signals were assigned by using the data for **10b** as reference. IR (KBr): $\tilde{\nu}$ = 3435 (ν NH), 2978 (ν_{as} CH$_2$), 2934 (ν_s CH$_2$), 1719 (ν C=O), 1622, 1497 (ν and δ ring), 1540 (δ NH), 1457 (ν C−N), 1395, 1363 (δ C(CH$_3$)$_3$], 1299, 1151 (ν C−O) cm^{-1}. $C_{30}H_{44}N_2O_{12}$ (624.7): calcd. C 57.68, H 7.10, N 4.48, found C 57.59, H 7.31, N 4.02

4-*O*-(α,4-Dicarboxy-2-nitrobenzyl) Aspartate (12a): A solution of **10a** (155 mg, 0.250 mmol) in a mixture of dichloromethane (12 mL) and trifluoroacetic acid (TFA, 4 mL) was stirred at room temp. under nitrogen for 16 h. The mixture was concentrated under reduced pressure, toluene (10 mL) was added, and the solvent was evaporated again. The procedure was repeated once and the residual solid was purified on Sephadex with water as eluent to give 78 mg (0.22 mmol, 88%) of a roughly 1.1:1 mixture of diastereomers of **12a** (estimated from the ^{1}H NMR integrals of the benzylic proton) as a white and very hygroscopic powdery solid. m.p. 128−130 °C. ^{1}H NMR (D_2O, 300 MHz): δ = 8.48 and 8.47 (2 d, $^4J_{H,H}$ = 1.8 Hz and 1.7 Hz, 1 H, Ar-3-H), 8.19 and 8.18 (2 dd, $^3J_{H,H}$ = 8.1 Hz, $^3J_{H,H}$ = 8.1, $^4J_{H,H}$ = 2.0, $^4J_{H,H}$ = 2.0 Hz, 1 H, Ar-5-H), 7.73 and 7.73 (2 d, $^3J_{H,H}$ = 8.2, $^3J_{H,H}$ = 8.2 Hz, 1 H, Ar-6-H), 6.73 and 6.70 (2s, (signal integral: 1.1:1), 1 H, α-H), 4.36 and 4.35 (2 dd, $^3J_{H,H}$ = 6.3, $^3J_{H,H}$ = 5.0 Hz and $^3J_{H,H}$ = 6.3, $^3J_{H,H}$ = 5.0 Hz 1 H, 2′-H), 3.300 and 3.296 and 3.22 and 3.21 (4 dd, $^2J_{H,H}$ = 18.0, $^3J_{H,H}$ = 6.4 Hz and $^2J_{H,H}$ = 18.0, $^3J_{H,H}$ = 5.1 Hz and $^2J_{H,H}$ = 18.1, $^3J_{H,H}$ = 6.4 Hz and $^2J_{H,H}$ = 18.1, $^3J_{H,H}$ = 5.1 Hz, 2 H, 3′-H). $C_{13}H_{12}N_2O_{10}\cdot 2H_2O$ (392.3): calcd. C 39.80, H 4.11, N 7.14, found C 39.60, H 4.08, N 6.97.

Photochemical Characterization

Transient Absorption: The transient absorption spectra were recorded with a setup described earlier.[16,22] Briefly, the caged compound was irradiated with light from a pulsed xenon chloride excimer laser (Lambda Physics EMG200, λ_{max} = 308 nm, pulse duration about 10 ns, about 50 mJ/pulse) in a quartz cuvette (2 × 10 mm path length, Hellma). The transient absorption was meas-

ured at a right angle to the incident laser light with a Xenon arc lamp (Zeiss), two monochromators (Optometrics Group) in front of and behind the sample, and a PIN photodiode (Siemens). The signal was preamplified (Advanced Research Instruments, PMT-4) and recorded and stored with a digital oscilloscope (LeCroy, 9310 AM, 6675 data points for each trace were stored). The data were evaluated by use of Microcal Origin v6.1. The measurements were conducted at 22 °C with solutions containing 1 mM of β-caged α,4-DCNB D-aspartate **12b** and 100 mM of the respective buffer. As buffer solutions, acetate (pH = 5.0), phosphate (pH = 6.0, 7.0, and 8.0), and borate (pH = 9.0 and 10.0) were used.

Quantum Yield: A joulemeter (Gentec) was added to the transient absorption spectrometer described above in order to measure the laser energy absorbed by the sample. Irradiation of the sample with laser pulses resulted in a reduction of the observed *aci*-nitro absorption with each consecutive laser pulse. On the assumptions that (i) the amount of released D-aspartic acid was directly proportional to the concentration of *aci*-nitro intermediate formed in each laser pulse, which is based on the proposed mechanism[37] and (ii) the absorption of the solution at 308 nm was constant during the measurement, which is validated by the experimental data, the quantum yield could be obtained from Equation (3), where A_n is the transient absorption of the *aci*-nitro intermediate at the *n*th laser pulse, ε_M the molar absorption coefficient of the *aci*-nitro intermediate, l the path length of the analysis light, ϕ the quantum yield, n_A the number of absorbed photons, V the total volume of the solution, F the fraction of the solution irradiated, c_0 the initial concentration of the caged compound, and n the number of the laser pulse.

$$\ln(A_n) = \ln\left(\varepsilon_M l \Phi \frac{n_A}{VF}\right) - \Phi \frac{n_A}{c_0 V}(n-1) \quad (3)$$

The quantum yield can be obtained directly from a plot of $\ln(A_n)$ versus $(n - 1)$ and only the amount of solution, the concentration of the solution, and the reduction of the laser energy by the sample have to be measured. In contrast to this, in order to measure the extinction coefficient of the intermediate, one has to obtain an estimate of the fraction of solution irradiated. This value can simply be estimated. The extinction coefficient has no influence on the determined quantum yields. The temperature, concentrations, and buffers used in these experiments were as mentioned above.

Ab initio Calculations

The ab initio calculations were carried out on a SGI Origin 2000 (IRIX 6.5.9) with the Gaussian 98[75] program package in the single processor mode. The STO-G 6-31G**[61−67] parameter set and the keywords opt, pop = full and gfinput were used. The charge was set to −1 (compounds **17**) or −2 (compounds **18** and **20**).

The semiempirical calculations were conducted on a Sun Workstation (SunOS 5.6) with the MOPAC 93 program package. The AM1 parameter set and the keywords T = 10000, ef, precise, vectors, charge −1 (compounds **17**) or −2 (compounds **18** and **19**) were used.

Cell Experimentation and Electrophysiology: HEK293 cell culture, transient transfection, and whole-cell current recording was performed as described earlier.[76] Briefly, subconfluent cells were transfected with EAAC1 cDNA at day 1 after the passage. Electrophysiological recordings were performed the day after transfection.

For whole-cell recordings, the extracellular solution contained (in mM) 140 NaCl, 2 $MgCl_2$, 2 $CaCl_2$, and 30 HEPES (pH 7.4/NaOH), while the pipette solution contained 130 KSCN, 2 $MgCl_2$, 10 TEACl, 10 EGTA, and 10 HEPES (pH 7.4/KOH). Typical pipette resistances were 2−3 MΩ, the series resistance was 5−6 MΩ. The experiments were carried out under voltage-clamp conditions with an Adams and List EPC7 amplifier.[77]

Rapid application of glutamate and caged aspartate to EAAC1-expressing cells was carried out with a solution-exchange device, as described earlier.[76] Photolysis of caged aspartate was initiated with a 345 nm light pulse from an excimer laser-pumped dye laser (Lambda Physics) which was delivered to the cell through an optical fiber (350 μm diameter) positioned in a distance of 300 μm from the cell.

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