Design and Synthesis of New Photolabile Protected Active Substances for Biophysical Investigations

Inaugural-Dissertation

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To**:**

MY MOTHER

and in memorial of:

MY FATHER

I kindly thank Prof. Dr. H. D. Martin for his friendly receipt in his study group and the liberty he granted to me during the studies for my “Promotion”. I highly appreciated his useful proposals and the fruitful discussions we had.

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I kindly thank Docent C. Grewer for the photochemical and biological measuring of my samples with MPI in Frankfurt. Some of the results would not have been achieved without his support.

List of abbreviations:

α- CNB	α-carboxy-2-nitrobenzyl
α-4,DCNB	α-4,dicarboxy-2-nitrobenzyl
α-5,DCNB	α-5,dicarboxy-2-nitrobenzyl
α-6,DCNB	α-6,dicarboxy-2-nitrobenzyl
ACh	acetylcholine
CNS	central nervous system
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	*N*,*N*-dicyclohexylcarbodiimide
EAAC1	excitatory amino acid carrier 1
EAAC3	excitatory amino acid carrier 3
GABA	γ-aminobutyric acid
GAD	L-glutamic acid decarboxylase
GLAST	glutamate aspartate transporter
HEK	human embryonic kidney
IPSP	inhibitory postsynaptic potential
NBS	*N*-bromosuccinimide
rt	room temperature
TFA	trifluoroacetic acid
TLC	thin layer chromatography

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1. Introduction

The nervous system provides for rapid communication between widely separated parts of the body. Through its role as communications network, it governs reactions to stimuli, processes information, and generates elaborate patterns of electrical signals to control complex behaviors. The nervous system is also capable of learning: as it processes and records sensory information about the external world, it undergoes adjustments that result in altered future pattern of action. At present, while the human brain as a whole remains the most baffling organ in the body (about 10^{11} nerve cells, with at least a thousand times that number of interactions), the properties of the individual nerve cells, or neurons, are understood better than those of any other cell type. At the cellular level at least, simple and general principles can be discerned. With their help, one can begin to see how small parts of the nervous system work. Knowledge of the molecular biology of neurons provides a key to the biochemical control of brain function through drugs, and it holds out the promise of more effective treatment for many forms of mental disease. Neurons in general are extremely elongated: a single nerve cell in human being, extended from the spinal cord to a muscle in the foot, may be more than a meter long. The fundamental task of a neuron is to receive, conduct and transmit signals (figure 1).

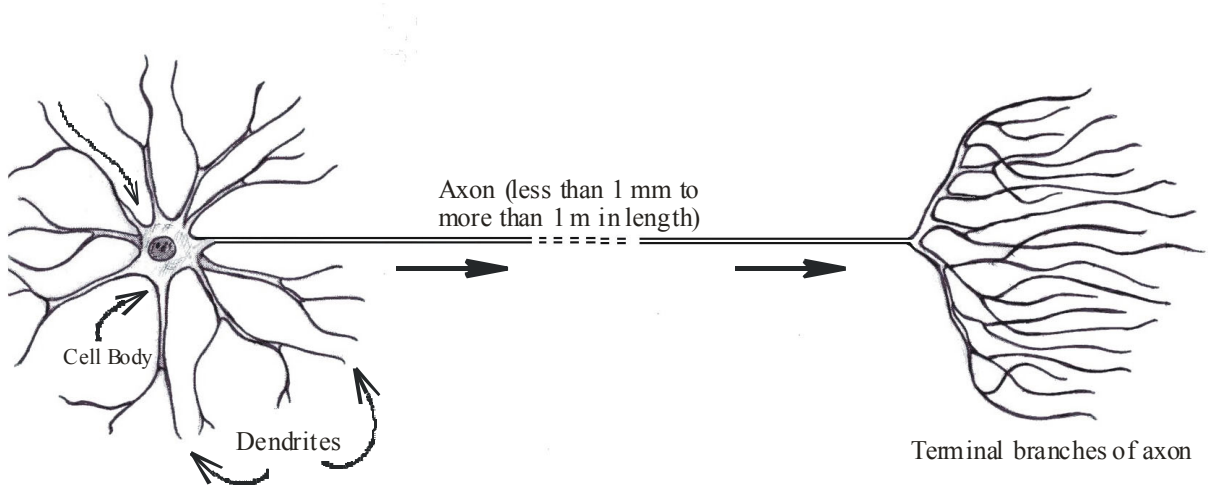


Figure 1: The neuron

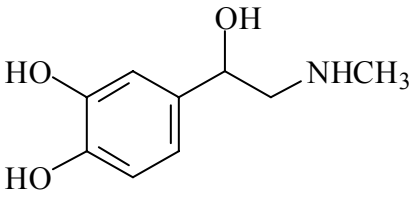
1.1 Historical Background

The hypothesis that neurons interact through chemical mediators is a complicated one. The general scheme is that an action potential in a presynaptic axon causes the release of a chemical that diffuses across the synaptic cleft to produce in the postsynaptic cell a change that leads either to excitation or inhibition. This process involves a metabolic machinery for the synthesis and storage of the chemical in the presynaptic neuron, a release mechanism, specialized in chemical sensitivity of the postsynaptic cell, and a mechanism whereby the chemical is inactivated.

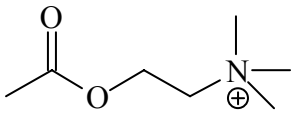
> *"If there exists any surface or separation at nexus between neurone and neurone, much of that is characteristic of the conduction exhibited by the reflex-arc might be more easily explainable . . . It seems therefore likely that the nexus between neurone and neurone in the reflex-arc, at least arc of the vertebrate, involves a surface of separation between neurone and neurone; and this as a transverse membrane across the conductor must be an important element in intercellular conduction. The characters distinguishing reflux-arc conduction from nerve-trunk conduction may therefore be largely due to intercellular barriers, delicate transverse membrane, in the former.*
>
> *In view, therefore, of the probable importance physiologically of this mode of nexus between neureone and neurone, it is convenient to have a term for it. The term introduced has been* **synapse***."*
>
> -Charles S. Sherrington, 1906[1]

The basic idea of chemical transmission between neurons originated from studies of the mammalian autonomic nervous system done early in this century. Langley (1906, 1907)[2, 3] noted the remarkable similarity between the effects of adrenalin (**A**), a naturally occurring substance isolated from the adrenal glands, and stimulation of neurons of the sympathetic nervous system. Both increase blood pressure and relax intestinal smooth muscles. Elliott[4] went so far as to suggest that "*Adrenalin might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery".* This splendid suggestion was not received with any great enthusiasm and possible reasons for this have been discussed by Dale[5-8]. He found that choline and its derivatives have effects similar to stimulation of

parasympathetic nerves on peripheral effectors such as the heart, bladder, and salivary glands. In particular, acetylcholine (**B**) was found to be the most potent. In these experiments the animal (dog, cat, rabbit, frog, etc.) was injected with a solution of the active substance and the corresponding reaction was observed (for example changes in behaviors of muscles or blood-pressure).



A



B

However, Loewi showed in his famous experiment that stimulation nerves leads to the release of an active chemical substance[9]. He collected the fluid perfusing a frog heart before and after stimulation of the vagus nerve. When applied to a second heart, the perfusate collected before vagal stimulation had no effect; but that collected during stimulation inhibited the beat of the second heart in the same way as the addition of acetylcholine or vagal stimulation. Dale and his colleagues[7, 10, 11] identified acetylcholine as the **neurotransmitter** at neuromuscular junctions.

Synaptic neurotransmitters are considered as substances that are released locally into an anatomically well-defined synaptic cleft, and influence the activity of only one or a few adjacent cells (figure 2).

Transmitters have been thought to produce rapid-onset and rapidly reversible responses in the target cell[12].

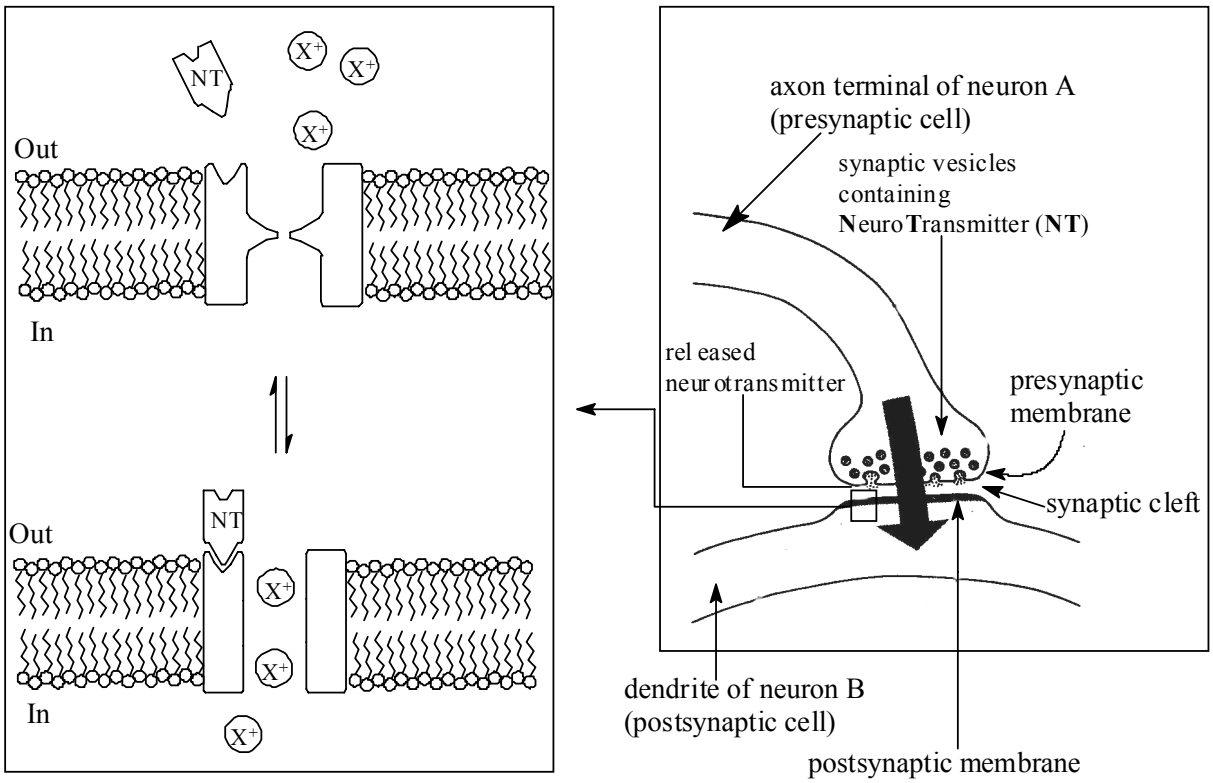
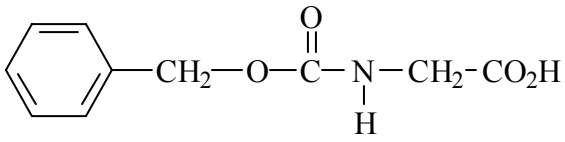


Figure 2: Synaptic cleft and release of the neurotransmitter

1.2 Chemical Perspectives and Overview

With the increased knowledge of the nature of photochemical processes and with sufficient instrumentation, preparative organic photochemistry has now been developed into an important branch of organic chemistry[13]. A newly developing field in which the principles of organic photochemical reactions are being advantageously exploited is in the design of photoremovable protecting groups for synthetic and biological purposes (Caged Compounds). The first report of a successful development of a photoremovable protecting group appears to be that of Barltrop and Schofield[14], who observed that benzyloxycarbonylglycine (**C**) is readily converted to the free amino acid by irradiation with ultraviolet light. Subsequently, a number of new photoremovable protecting groups have been developed for various functional groups[15-37].



C

The term “caged” was originally coined by Kaplan *et al*[15] (1978) to describe the unique properties of a 1-(2-nitrophenyl)ethyl ester of ATP. This ATP analogue permitted a biological system to be pre-equilibrated with ATP in an inactive form. The system could then be activated by rapidly converting caged ATP to ATP using near-UV irradiation.

The principal application of caged compounds has been time resolved measurements with physiological preparations that are not suited to rapid mixing techniques. Caged compounds photolyse with half-times in the nano to millisecond[38, 39] time range and thus can be used to overcome limitations imposed by diffusion, uptake, or metabolism of a biological substrate in a complex preparation. Another important advantage of light activation is that light beams are easily shaped and directed. As a result, photolysis can be effected in[40-42] or on the surface[16-20, 43-46] of a single cell, in principle, in a selected region. Photolysis can be used to initiate reactions within intact living cells.

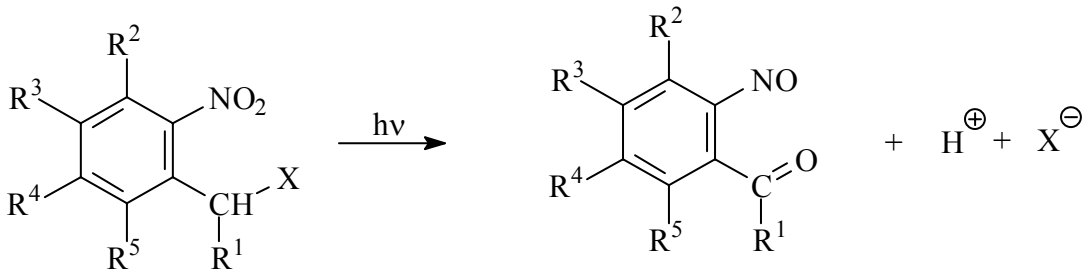
For biophysical or pharmacological studies, one requires a photosensitive molecule that satisfies several criteria[47].

1. The compound must be soluble in aqueous solutions of moderately high ionic strength.
2. The photochemical reaction must also proceed in this medium.
3. The photochemical reaction should take place instantaneously with respect to the time scale (a few μs) of the physiological phenomenon being studied, without the formation of any reactive intermediates or active by-products.
4. The photoproduct should be stable (thermally and solvolytically).
5. The photochemical reaction should be initiated at wavelengths long enough to cause no significant damage to cellular compounds ($\lambda > 320$).
6. The efficiency per incident photon should place the reaction within reach of flashlamps and pulsed lasers (high quantum yields).

7. Both the precursor and the photoproduct should have simple, well characterized effects on the physiological system; there should be no complicating interactions with proteins or membranes.

Most of the caged compounds in common usage rely on the photochemistry of the 2-nitrobenzyl group. The light-induced internal oxidation-reduction reactions of these compounds containing a carbon-hydrogen bond at the benzyl position have been the subject of many investigations[48, 49]. The photochemical removal of the 2-nitrobenzyl group was first observed in the irradiation of 2-nitrobenzyl benzoate (scheme 1: **1**; R^1-R^5 = H, X = PhCOO).

Scheme 1:



1 **2**

In this case benzoic acid was formed only in 17% yield[50]. To modify the photolytic cleavage of the 2-nitrobenzyl group, substituted 2-nitrobenzyl esters were used[51]. Substitutions on both the aromatic ring and the benzyl carbon have been made in order to improve photochemical and biological properties. A brief discussion of substituent effects will be given mainly to explain why certain compounds are preferred over others.

A methyl group on the benzyl carbon (scheme 1: **1**; $R^1 = CH_3$) is thought to confer two advantages to a caged compound: an increased rate of photolysis and a lessening of the reactivity of the photolysis by-product. Substitution of a benzyl proton with a methyl group increases the rate of caged charbamoyl choline photolysis by 20-fold[16]. The same substitution in caged ATP increases its photolysis rate and increases the quantum yield[15]. Without the methyl group the nitrosobenzaldehyde by-product apparently reacts with the adenine moiety of ATP reducing the yield of free ATP. Another common modification of the 2-nitrobenzyl moiety is the substitution of various positions of the phenyl ring. The 4,5-

dimethoxy caged cyclic nucleotides (scheme 1: $R^3 = R^4 = OCH_3$, $R^1 = R^2 = R^5 =$ H) show a dramatic increase in photolysis rate, but there is almost an equally dramatic decrease in the quantum yield[52, 53].

Another reason for altering substituents on the photolabile group is to modify the biological properties of a caged compound. An early version of caged carbamoyl choline consisted of a 1-(2-nitrophenyl) ethyl group linked to the carbamate nitrogen atom (scheme 1, $R^1 = CH_3$, $R^2 = R^5 = H$, X = *N*-carbamoylcholine). While the photochemistry of this compound is highly favorable, it is not biologically inactive in preparations containing nicotinic acetylcholine receptors. This problem has been solved by introducing a carboxylate group into the benzyl carbon to increase the hydrophilic character of that portion of the molecule. The α-carboxylate substituted compound also has improved photochemical properties making it the caged carbamoylcholine of choice[16]. Introduction of a carboxylate group would be one way of changing membrane permeating caged compounds such as caged cyclic nucleotides into not membrane permeating compounds that could then be localized in aqueous compartments within a cell. The compounds caged with the α-carboxy-*o*-nitrobenzyl (α-CNB) group that are not membrane permeating are carbamoylcholine[16] and the inhibitory neurotransmitter γ-aminobutyric acid (GABA)[54] and the excitatory* neurotransmitters glutamate[45] and kainic acid[19]. The photolysis of these caged neurotransmitters generates the free amino acids in 20-80 μsec with quantum yields of 0.15-0.43. Such rapid activation allows these compounds to be used in the study of fast receptor activation[16, 55], inhibition[56-58] and the desensitization steps[16, 59, 60].

Neurotransmitters are very diverse and have been divided into three categories.

1 *Type I* neurotransmitters are simple amino acids such as L-glutamic acid and γ-aminobutyric acid GABA (in their anion forms: *L-glutamate*, *γ-aminobutyrate*) and *glycine* which may account for transmission at up to 90% of all CNS (central nervous system) synapses and are responsible for fast information transfer. These neurotransmitters are present in the brain in micromoles per gram wet weight.

* If the binding of a transmitter to a receptor increases the likelihood of the cell firing a nerve impulse, then the receptor is said to be *excitatory*. Conversely activation of an *inhibitory* receptor will decrease the likelihood of firing.

2 *Type II* neurotransmitters include the "classical" transmitters *acetylcholine (ACh)*, the catecholamines (*dopamine, noradrenaline* and to a lesser extent *adrenaline*) and *5-hydroxytryptamine (5-HT)*. These are present in most areas of brain at nanomoles per gram wet weight. Type II transmitters are predominately slow acting and play a modulatory role in CNS.

3 *Type III* neurotransmitters encompass a wide variety of *neuropeptides* which are characteristically present at very low concentrations (nanomoles to picomoles per gram of brain). Neuropeptides act predominantly as *neuromodulators* controlling the activity of diffuse receptors rather than at purely synaptic sites.

Some of them are summarized in table **1**[61].

Type		Appropriate levels in rat brain ($nmol.g^{-1}$)
Type I: amino acids	Glutamate* (Aspartate)* γ-Aminobutyrate Glycine*	14 000 4 000 2 500 2 000
Type II: amines and purines	Acetylcholine Dopamine Noradrenaline Histamine*	25 6.5 2.5 1
Type III: Peptides	[Met]-enkephalin ◊ Substance P §	0.35 0.1

Table 1: Major classes of neurotransmitters. * A major proportion is in non-transmitter pools, ◊ Tyr-Gly-Gly-Phe-Met, § Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met.

1.3 Importance of the L-Glutamate and the γ-Aminobutyric Acid in CNS

The possibility that L-glutamate may have a special significance in brain function was assumed more than 50 years ago[62]. The suggestion that L-glutamate may actually be a synaptic transmitter substance in brain was first made by Hayashi in 1954[63]. At about the same time, techniques for investigating such functions of a substance in the CNS were just being developed, and it was soon realized that the two major neurotransmitters of the peripheral nervous system, actylcholine and noradrenaline, must play a much less important

role in the CNS[64]. When in 1959[65], it was found that L-glutamate depolarized and excited individual neurons in the cat spinal cord, hopes were raised that this substance was indeed a neurotransmitter in the CNS. This possibility was accepted 20 years later[45, 66-70].

GABA (γ-aminobuyric acid) is the major inhibitory neurotransmitter in the central nervous system (glycine mediates most of the rest of the inhibitory synapses *in the body*) and is secreted by neurons in the spinal cord, basal ganglia and cortex. Its precursor is glutamate and GAD (L-glutamic acid decarboxylase) catalyzes the conversion. The accumulating GABA concentrations in the synaptic clefts regulate the activity of GAD via feedback inhibition. GABA production and release is stimulated by depolarization, and is also calcium-dependent. The activity of this inhibitory neurotransmitter is terminated by selective uptake into the presynaptic terminals and glial cells*. In the glial cells GABA is metabolized by GABA transaminase to succinic semialdehyde. After a series of reactions, it is then oxidized to succinic acid and thus enters the citric acid cycle of the glial cells.

GABA mediates presynaptic inhibition in the spinal cord and fast IPSPs (inhibitory postsynaptic potentials) in the brain. Presynaptic inhibition occurs in the presynaptic terminals before the signal reaches the synapse. GABA opens chloride channels and the flood of chloride ions into the terminal reduces the action potential which decreases the degree of excitation of the postsynaptic neuron. An IPSP also works by opening chloride channels, so chloride ions move into postsynaptic nerve cell and cause hyperpolarization; the membrane potential is further away from the activation threshold.

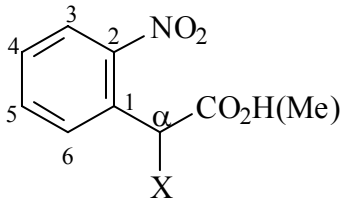
2. Objective

Improvement of the hydrophilicity of the caged compounds through introduction of polar groups into the aromatic ring is the chief goal of this dissertation. This work consists of two main parts: 1. Synthesis of the new caged compounds and 2. Measurement and investigation of the photochemical and biophysical properties of the synthesized substances.

* Glial cells, which constitute the majority of cells in the nervous system, act as intermediaries between blood vessels and neurons, providing the neurons with mechanical support, nutrients, and other substances that may enhance neuronal function.

In order to improve the water solubility of the caged compounds, an additional carboxy group should be introduced in the aromatic ring (scheme 2).

Scheme 2:



In most of our biological investigations potassium thiocyanate is used. This ion can pass through the ion channels, therefore our work aimed at the synthesis and investigation of some caged thiocyanates.

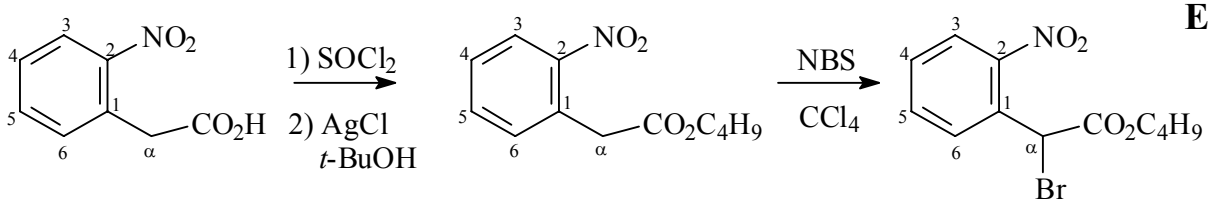
3. Results and Discussion

3.1 Synthesis

3.1.1 Syntheses of the Building Blocks

For the preparation of the α-carboxy-(2-nitrobenzyl) protecting group commercially available 2-nitrophenyl acetic acid has been used. In two simple steps compound **5a** was prepared by an improved method analogous to a procedure described by Takimoto[71, 72] (scheme 3).

Scheme 3:

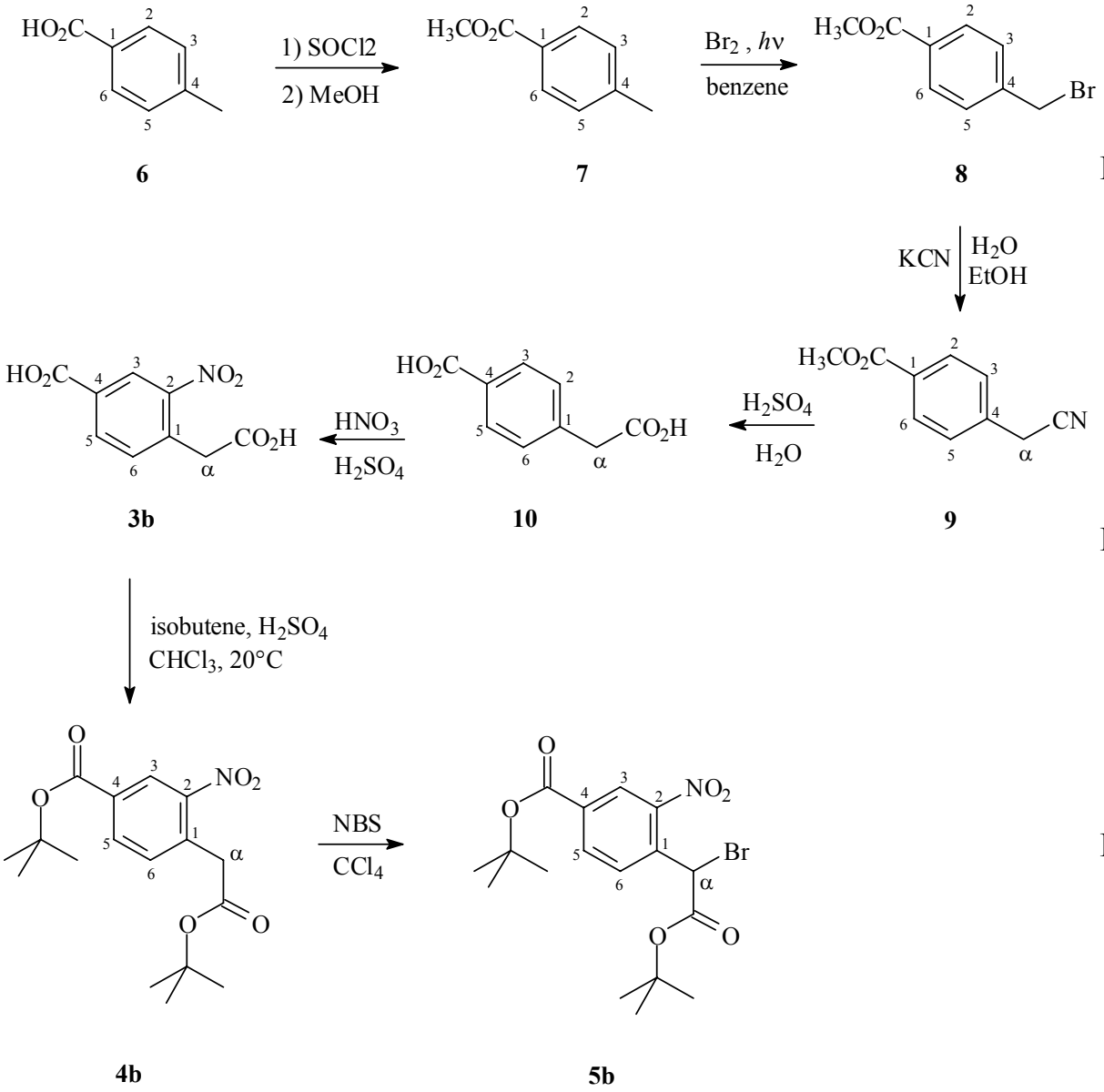


3a **4a** **5a**

E1, E2

To introduce a carboxy group into the 4-position of the aromatic ring, *p*-toluic acid **6**, was used. In four steps, (*p*-carboxyphenyl)acetic acid **10** was obtained by standard methods[73-76]. Selective nitration in the 3-position yielded the dicarboxylic acid **3b**[77], which was then double esterified with isobutene[78] in two weeks. Finally, bromination yielded the desired protecting group **5b**[76] (scheme 4).

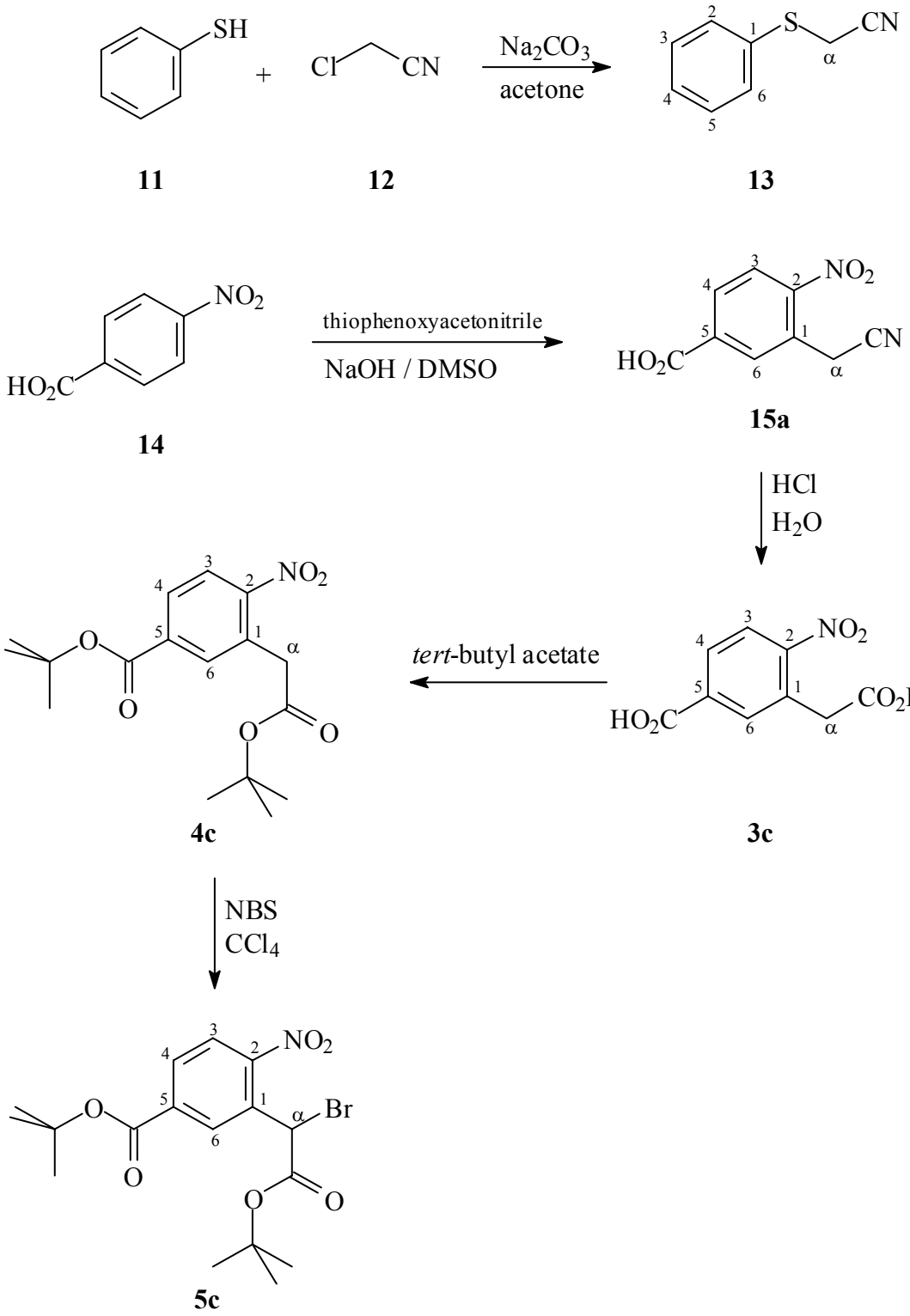
Scheme 4:



In order to synthesize a dicarboxylic acid with an additional carboxy group in 5-position p-nitrobenzoic acid **14** was used as starting material. Introducing a cyanomethyl group via the "Vicarious Nucleophilic Substitution" in the 3-position of this species with thiophenoxyacetonitrile[79] has been described by Makosza[80]. Apart from the esterification,

similar procedures as in the synthesis of **5b** have been used. This procedure yields similar results as the one described above, but it has the advantage, that it requires less synthetic steps (scheme 5).

Scheme 5:



To synthesize of the 6-carboxy derivate (scheme 6), nitration of the appropriate dicarboxilic acid was not suitable (because a product mixture is expected) and therefore, the protecting group **3d** has been synthesized by a similar method as **3b,** using 2-nitro-3-methyl benzoic acid **16** as starting material. Although the bromide **5d** could be synthesized, substitution of the bromide with various reagents (e. g. carboxylates, thiocyanate ion and amines) was not possible (schemes 6). (For these reactions either the amine moiety of glycine was protected as BOC **5e** or the carboxy function as its *tert*-butyl ester **5h** as described in literature[81, 82]). Calculations on AM1 level show that the benzylic carbon (α-C) in **5d** is sterically hindered through *tert*-butyl groups and the bromine atom. Therefore it is not possible this atom to be attacked by a nucleophile (figure 3, calculations in appendix IV).

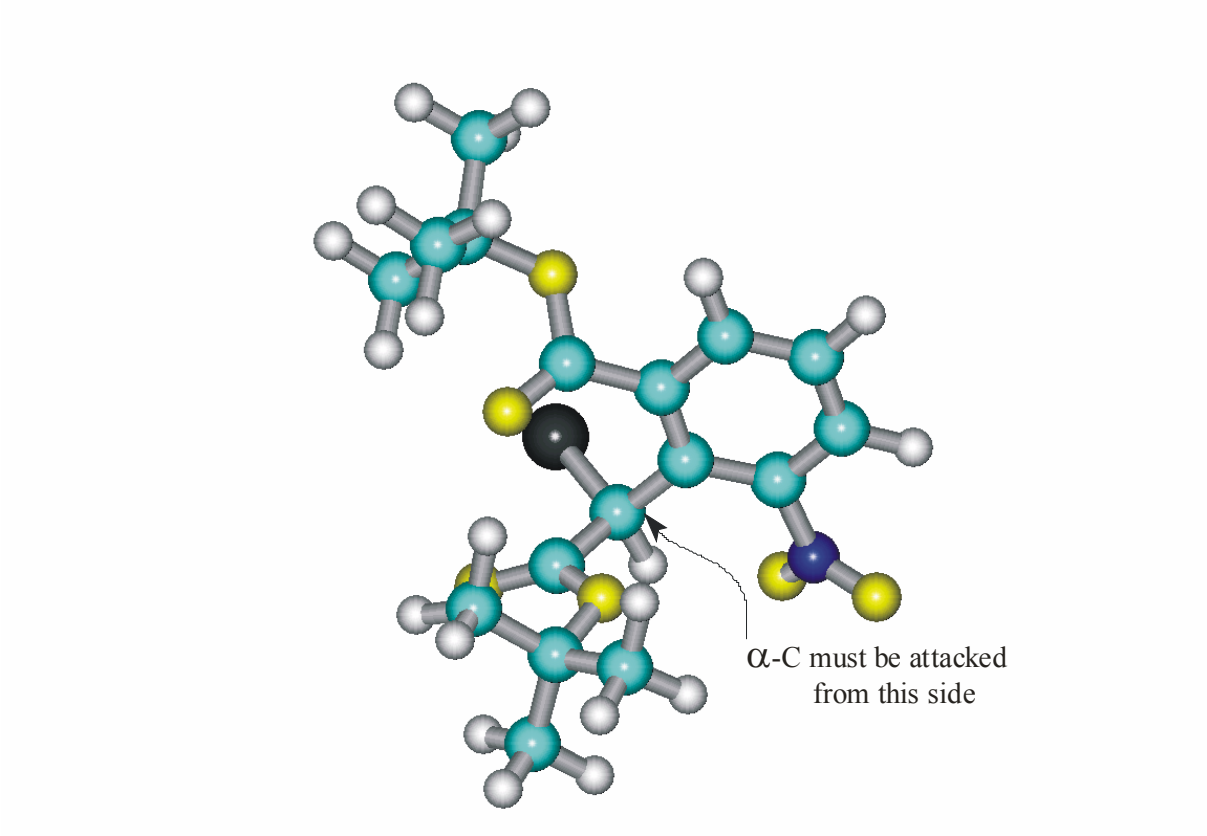
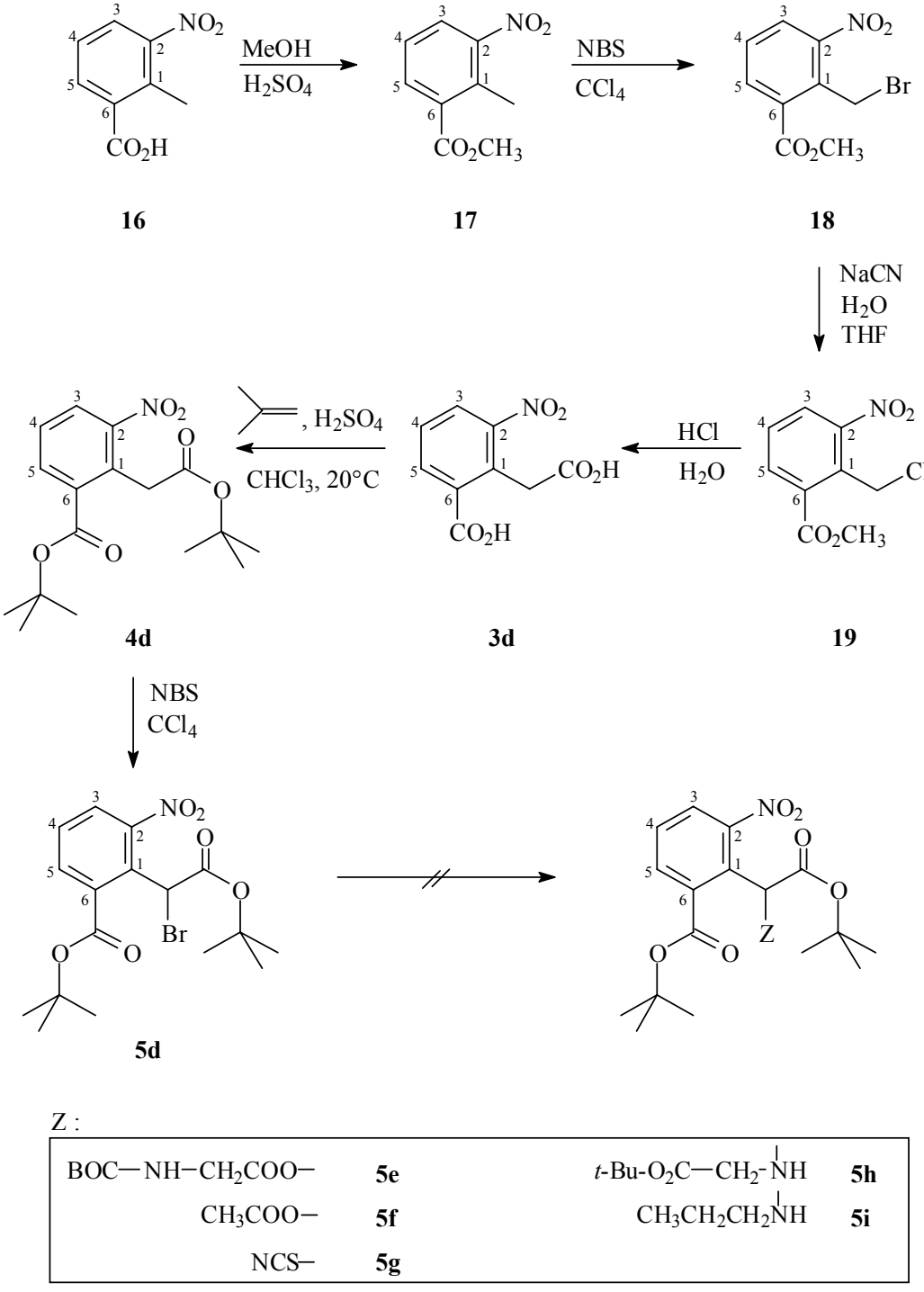


Figure 3: the structure of **5d** after AM1 calculations

Due to this, the synthesis of the analogue dimethyl ester **21c** was thought.

Since the deprotection of the methyl groups from the ester without damaging the cyanate moiety is not possible, this substance as an ester is not water soluble, but these substances are theoretically interesting and therefore we have synthesized **21b** and **21c** (precursors of the caged compounds **41** and **42**) and to evaluate their photochemical properties, **21a (**precursor of the **39**) is prepared as reference (scheme 7).

Scheme 6:



E15, E16

E17- E19

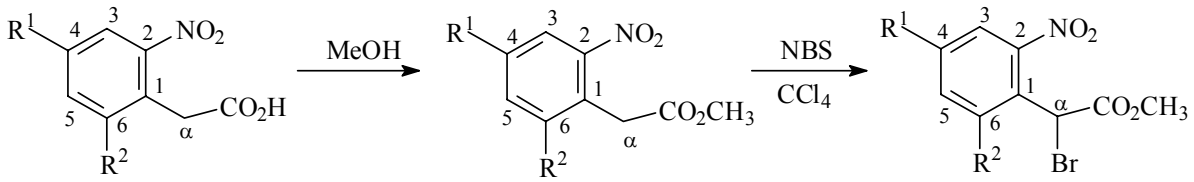
E20

Z :

BOC—NH—CH_2COO–	**5e**	*t*-Bu-O_2C—CH_2-NH	**5h**
CH_3COO–	**5f**	$CH_3CH_2CH_2$NH	**5i**
NCS–	**5g**		

Because of the hygroscopic nature of **3b-d** and **15a**, it was not possible to obtain their elemental analysis, and therefore they have been converted into their methyl esters **20b-d**, and **15b** as well[83] (schemes 7, 8 and 9).

Scheme 7:

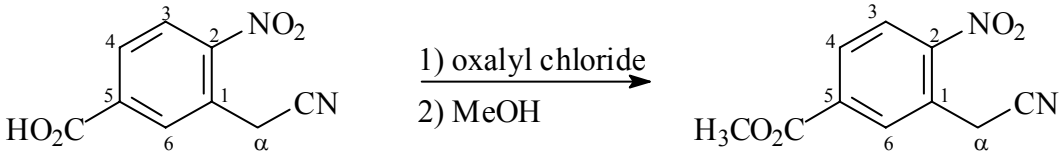


E21- E26

3a-d

$R^1 = R^2 = H$	**20a**	$R^1 = R^2 = H$	**21a**
$R^1 = CO_2Me$, $R^2 = H$	**20b**	$R^1 = CO_2Me$, $R^2 = H$	**21b**
$R^2 = CO_2Me$, $R^1 = H$	**20c**	$R^2 = CO_2Me$, $R^1 = H$	**21c**

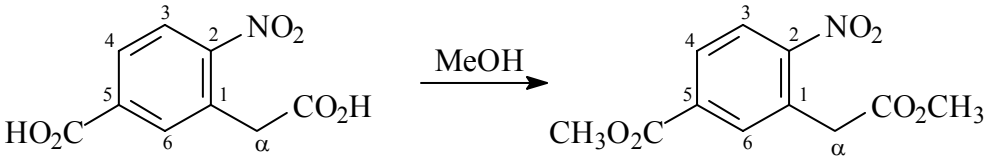
Scheme 8:



E27

15a **15b**

Scheme 9:

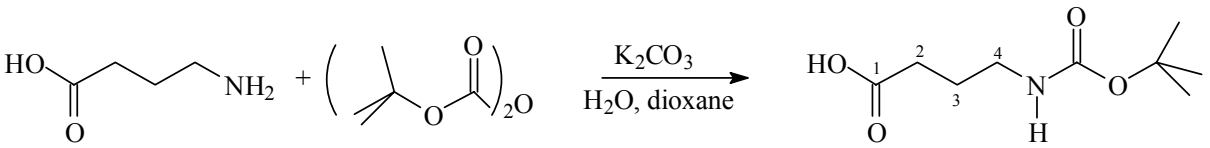


E28

3c **20d**

As building block for further synthesis, γ-aminobutyric acid (GABA) was protected in a quantitative reaction at the amino group with di-*tert*-butoxy-orthocarbonate (di-BOC) in one step[81] (scheme 10).

Scheme 10:



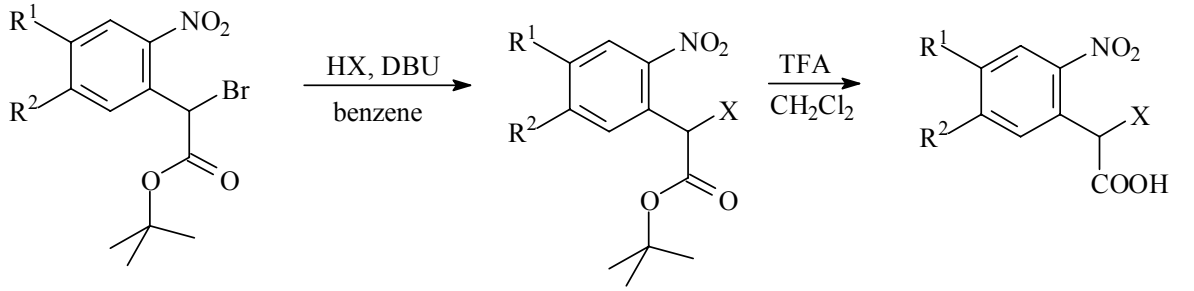
22 **23** **24**

E29

3.1.2 Syntheses of the Caged Compounds

The substitution of the bromine atom of the protecting group (with DBU as base[84]) was the general method to synthesize caged compounds (**37-41**, scheme 12) or their *tert*-butyl and BOC protected precursor (**25-30**, scheme 11). In next step, for the precursors (**25-30**), the *tert*-butyl groups were later removed with trifluoroacetic acid (TFA) in dichloromethane.

Scheme 11:



5a-c

X = Asp[a] :
$R^1 = R^2 = H$ **25**
$R^1 = CO_2$-*tert*-Bu, $R^2 = H$ **26**
$R^2 = CO_2$-*tert*-Bu, $R^1 = H$ **27**

X = O_2CCH_3:
$R^2 = CO_2$-*tert*-Bu, $R^1 = H$ **27a**

X = glut[b] :
$R^1 = CO_2$-*tert*-Bu, $R^2 = H$ **28**

X = GABA[c] :
$R^1 = CO_2$-*tert*-Bu, $R^2 = H$ **29**

X = Tol[d] :
$R^1 = R^2 = H$ **30**

X = Asp[e] :
$R^1 = R^2 = H$ **31**
$R^1 = CO_2H$, $R^2 = H$ **32**
$R^2 = CO_2H$, $R^1 = H$ **33**

E30- E36

X = glut[f] :
$R^1 = CO_2H$, $R^2 = H$ **34**

E37- E42

X = GABA[g] :
$R^1 = CO_2H$, $R^2 = H$ **35**

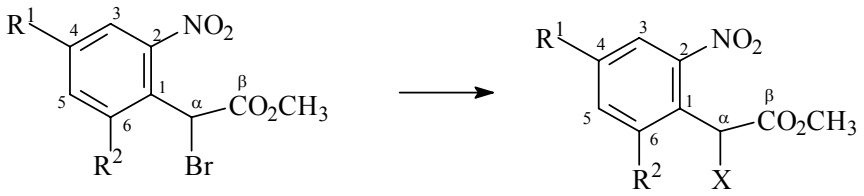
X = Tol[d] :
$R^1 = R^2 = H$ **36a**

a: α-*tert*-butyl ester of *N*-BOC -D-aspartate
b: α-*tert*-butyl ester of *N*-BOC -L-glutamate
c: *N*-BOC-γ-aminobutyrate
d: 4-methylbenzoate
e: β-D-aspartate
f: γ-D-glutamate
g: γ-aminobutyrate

The deprotection of the *tert*-butyl ester to water soluble caged thiocyanates was not successful and since all trials to substitute the bromine atom of the *tert*-butyl ester of the protecting group **5d** failed, we prepared methyl protected caged thiocyanates (scheme 12).

To investigate the photochemical characteristics of the α,6-DCNB, caged acetate **38** was synthesized. Additionally, the acetate **37** was synthesized as reference compound for **38** (scheme 12).

Scheme 12:



21a, b, d

X = -OOCCH$_3$ (OAc):

$R^1 = R_2 = H$	**37**	**E43- E44**
$R^1 = H, R^2 = CO_2Me$	**38**	

X = -SCN:

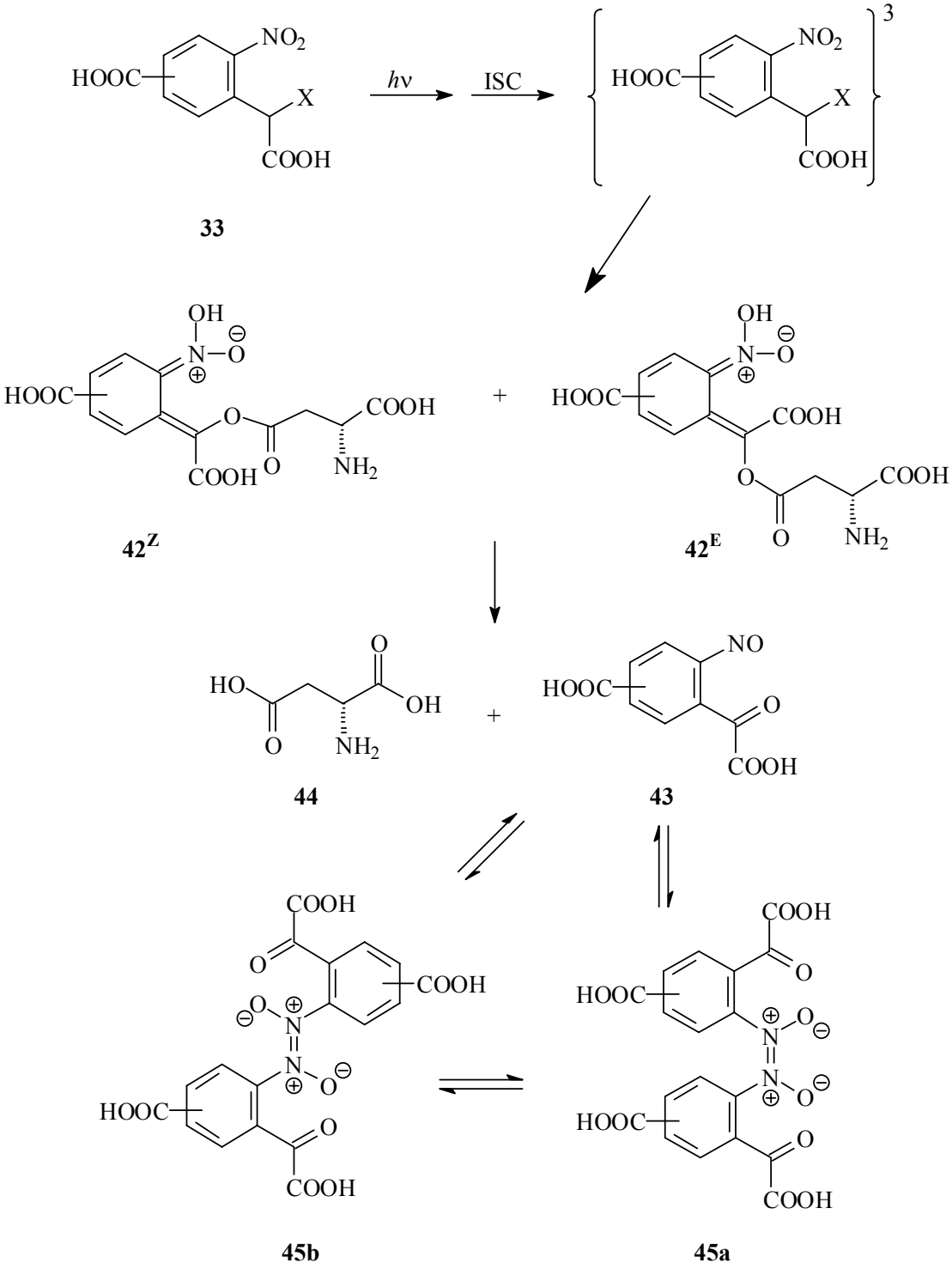
$R^1 = R^2 = H$	**39**	
$R^1 = CO_2Me, R^2 = H$	**40**	**E45- E47**
$R^1 = H, R^2 = CO_2Me$	**41**	

3.2 Photochemical Characterization

The caged compounds with α-CNB[72], α,4-DCNB[76] and α,5-DCNB caging groups were investigated by transient UV spectroscopy. For compounds with α,6-DCNB caging group the final deprotection of the carboxyl groups failed, therefore for those compounds the dimethyl esters were investigated.

Since these caged compounds show similar properties, first the photochemical and physiological characteristics of the *α*-CNB-D-aspartate **31** and α,4-DCNB-D-aspartate **32** will be reported in detail. The results of the other photochemical investigations will be summarized in tables and appendixes. We have predicted that α,5-DCNB must have better photochemical properties[76] than α,4-DCNB, this was proved by our measurements for **33.**

Scheme 13



3.2.1 Kinetic Investigations:

The characteristics of the photochemical reactions of the caged compounds were evaluated with transient UV-vis absorption spectroscopy after initiation of the reaction by a pulse of UV light. Figure 4 shows the transient absorbance produced by a 10 ns 308 nm pulse of laser light

in a solution of **32** (for **31** and **33** see appendix I, figure 1 and appendix III, figure 1). The absorption is caused by transient formation of the *aci*-nitro intermediate **42** (scheme 13) with a maximum absorption at about 430 nm. The decay of the *aci*-nitro intermediate is a two-exponential process with the parameters: $A_0 = 0.03$, $A_1 = 0.66$, $k_1 = 9050 \pm 58\ s^{-1}$, $A_2 = 0.31$, $k_2 = 1970 \pm 500\ s^{-1}$, total amplitude ($A_{Tot}$) normalized to 1 (data from the absorption spectrum of **32**). This behavior is typical for the photochemistry of *o*-nitrobenzyl compounds[18-20, 72, 85, 86]. According to earlier proposals[87-89] the existence of the two kinetic components can be most likely attributed to the possible stereoisomerism of the *aci*-nitro intermediate, which can be formed as *Z*-isomer **42**$^{\mathbf{Z}}$ or *E*-isomer **42**$^{\mathbf{E}}$[72] (scheme 13). The observed rate constant of the fast phase is about 1/4 of the rate constant for the respective reaction of **31** ($k_1 = (3.85 \pm 0.40) * 10^4\ s^{-1}$). The slow and minor phases of the reaction decay with the same rate within experimental error (for **31** $k_2 = 2400 \pm 310\ s^{-1}$). Therefore, the improved hydrophilicity is linked to a somehow slower photo release of the active compound.

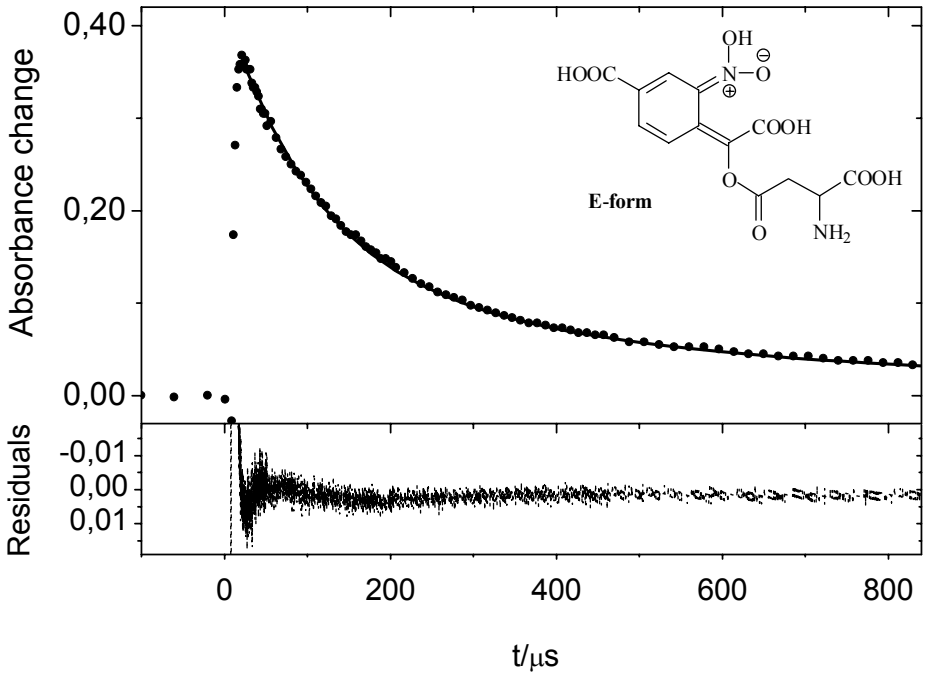


Figure 4: *aci*-Nitro intermediate decay produced by photolysis of 1 mM β-caged α,4-DCNB, **32** in 100 mM phosphate buffer (pH = 7). A 10 ns laser flash at 308 nm was given at time t = 0 s to initiate the reaction, and the absorption was measured at 430 nm. The solid line presents the best fit to the data ($A_0 = 0.013$, $A_1 = 0.27$, $t_1 = 110$ μs, $A_2 = 0.13$, $t_2 = 508$ μs), the filled circles represent the measured data; the lower panel shows the residuals of the fit.

In a similar experiment α,5-CNB-D-aspartate **33** was used. The measured rate constants are in good agreement with the predicted properties for **33**[76]. The results lead within experimental error to a linear correlation between the observed rate constants and the calculated Klopman energies[90] ΔE_{Tot} (table 1 and figure 5).

Compound	$t_1/\mu s$	$\Delta E_{Tot}/\frac{kJ}{mol}$	$t_2/\mu s$	$\Delta E_{Tot}/\frac{kJ}{mol}$
X	D-aspartate	OAc	D-aspartate	OAc
31	29 (80%)	-94.2	308 (20%)	-90.3
32	110 (72%)	-84.5	508 (28%)	-79.6
33	56 (89%)	-91.8	240 (11%)	-82.8

Table 1: Observed time constants and Klopman energies for the compounds **31**-**33** are shown.

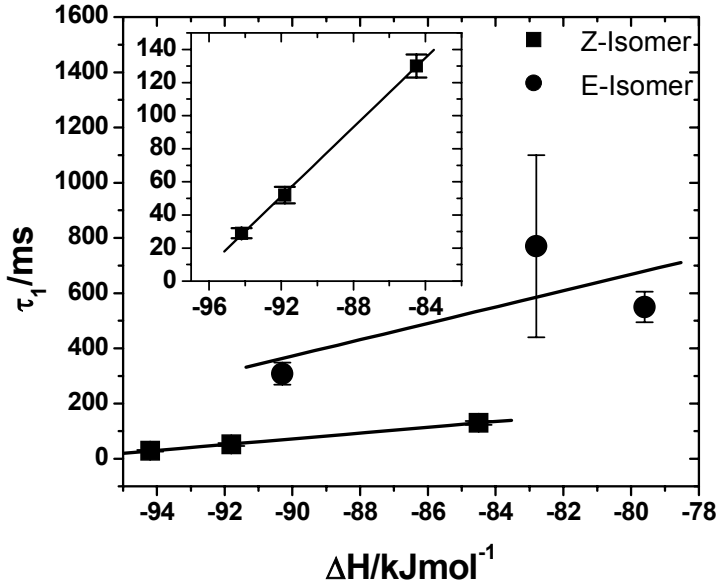


Figure 5. A Comparison of the Klopman energies and the observed time constants for compounds **31**-**33** (see table 1) is shown.

Therefore the caged compound **33** combines the rapid release of the active compound with an increased hydrophilicity and presents a major improvement in companion to the nitrobenzyl caging groups used so far.

The four absorption spectra in figure 6 are constructed from absorbance values determined at 0.500 µs, 100 µs, 400 µs, 2 ms after the laser flash from the *aci*-nitro decay measured at several wavelengths between 350 nm and 620 nm (irradiation of **32**). The absorption maximum of this intermediate is about 460 nm. In comparison with the typical absorption of *aci*-nitro intermediates seen with the α-CNB protecting group (appendix I, figure 2), the absorption is red shifted by about 30 nm.

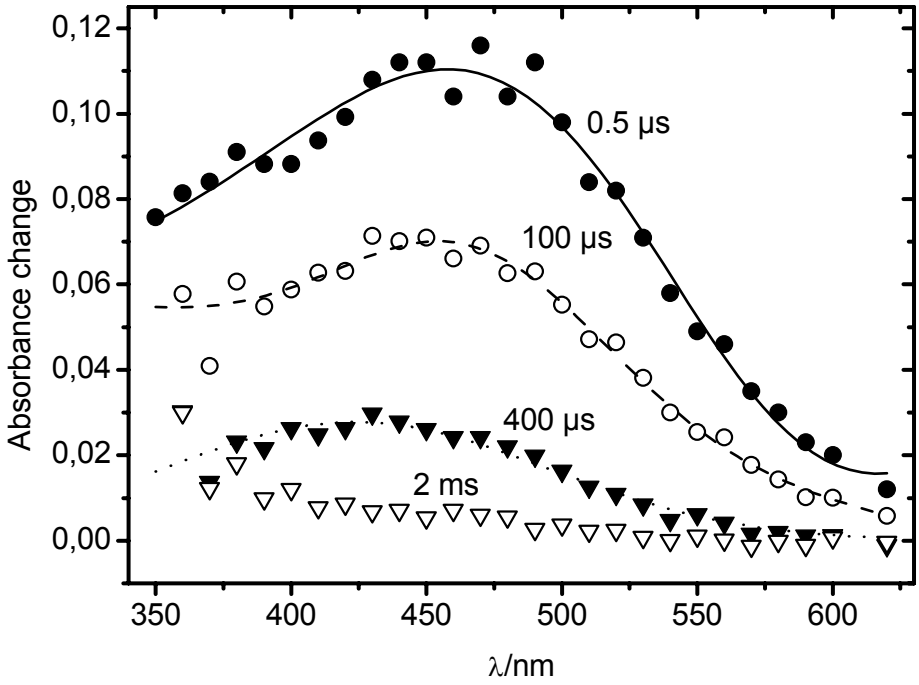


Figure 6: Spectral distribution of the transient absorbance recorded at: 0.5 µs, 100 µs, 400 µs and 2 ms after the laser flash on **32**.

Irradiation of a sample of α-CNB-D-aspartate **(31)** with 100 consecutive laser pulses leads to complete photolysis. The spectrum shows an increase of the absorbance at wavelengths higher than approximately 300 nm, which is typical for photoproducts associated with the 2-nitrobenzyl photochemistry[16, 91]. The typical spectral change is shown in figure 7. The characteristic absorption at 320 nm[92] is caused by the formation of the azodioxide[93] **45**, the dimer of the nitroso-compound. The typical absorption of the nitroso-monomer around 750 nm has a very low extinction coefficient and could not be observed. The small but distinct absorption of **45** at 430 nm causes the observed offset (A_0) in the transient absorption spectra (figure 4).

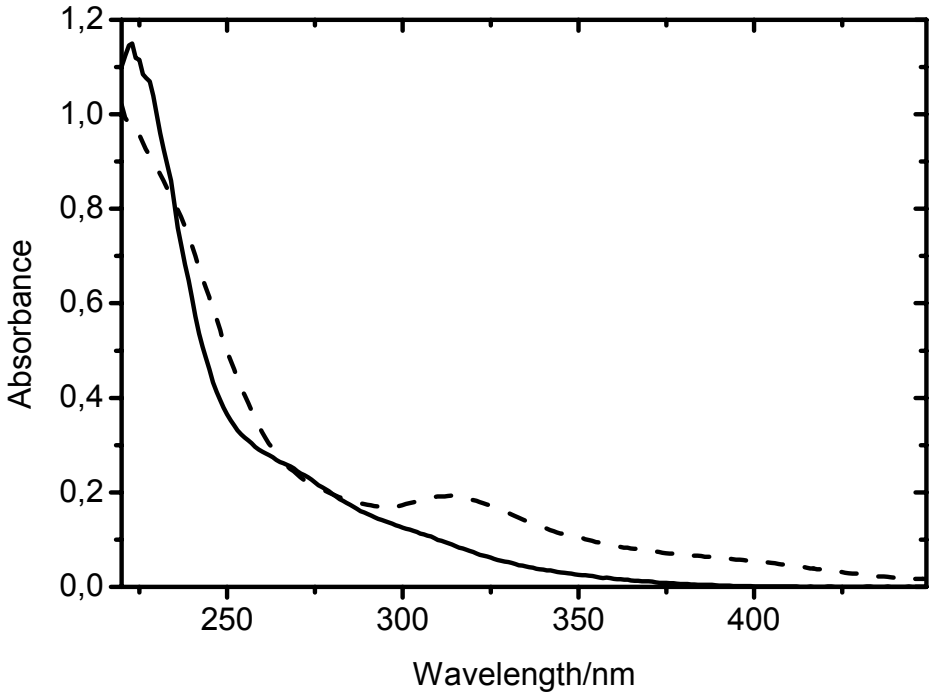


Figure 7: UV-visible spectrum of α-CNB-D-aspartate **31** (50 μM, 100 mM phosphate buffer, pH = 7.1) before photolysis (straight line) and of the reaction products (dashed line) after complete hydrolysis (100 laser pulses of 15 mJ, 330 μl solution).

3.2.2 pH Dependence of the Photolysis Reaction

The glutamate transport process is highly pH dependent[69, 70]. Therefore, the pH dependence of the photolysis reaction was investigated to test if the new caged derivatives can be used at a pH much different from 7.0. Therefore, the kinetics of the decay of the *aci*-nitro intermediate was measured at different pH values. As shown in figure 8 and summarized in table 2, in case of α,4-DCNB-D-aspartate **32** both rate constants demonstrated a significant pH-dependence, they increase with decreasing pH in the range between 5 and 10 (acid catalysis). [the rate constant for the slowly decaying of **31** in pH-range between 6 and 9 is pH-independent[72] (appendix I, table 1 and figure 3).] A decrease in the rate constant around pH = 5 has been reported for caged compounds with an α-carboxy group[18, 19]. This behaviour is in accordance with theoretical investigations, which attributed the increased rate at lower pH to the protonation of *aci*-nitro group and the decreased rate at pH < 5 to protonation of the α-carboxy group[89].

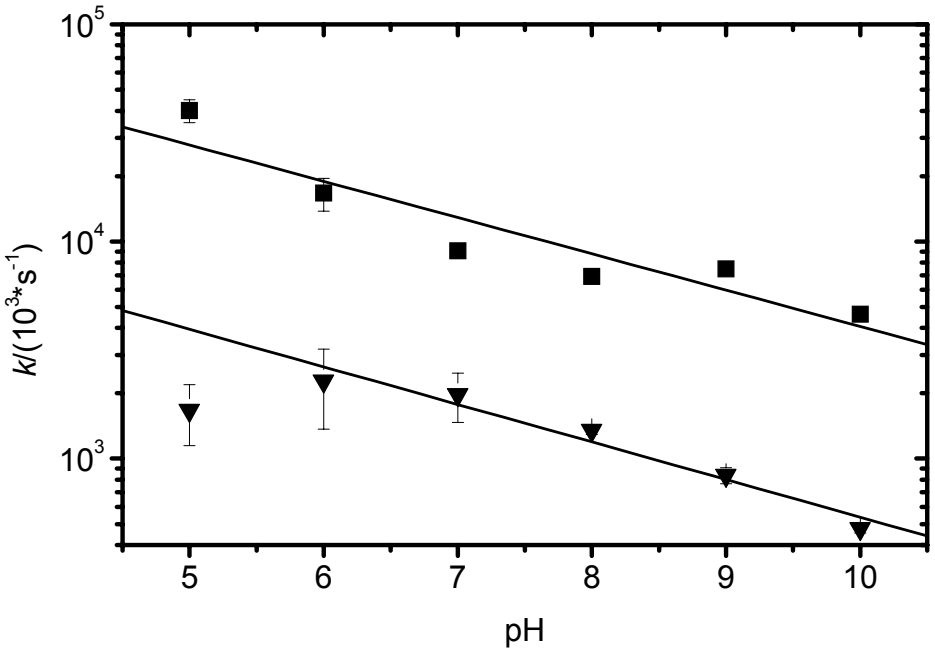


Figure 8: pH-Dependence of the rate constants of the fast (k_1) and the slow (k_2) exponential component of the decay of the *aci*-nitro intermediates **42** of the **32**.

pH	$k_1/(10^3 s^{-1})$	$k_2/(10^3 s^{-1})$
5	41.1 ± 4.9	1.67 ± 0.52
6	16.7 ± 2.9	2.28 ± 0.91
7	9.05 ± 0.05	1.97 ± 0.50
8	6.89 ± 0.38	1.35 ± 0.06
9	7.49 ± 0.30	0.837 ± 0.07
10	4.63 ± 0.33	0.477 ± 0.02

Table 2: The rate constants for the decay of the *aci*-nitro intermediate, formed from the α,4-DCNB-D-aspartate **32** upon irradiation with a laser at 308 nm at different pH are given. 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 from transient UV-vis spectroscopy at 430 nm.

3.2.3 Quantum Yield

The setup to determine the kinetic properties of the caged compounds is shown in figure 9. The compound is photolysed with a laser pulse from an eximer gas laser. The transient absorption is determined at right angle to the laser beam. The absorption of laser light is measured additionally in order to determine the quantum yield.

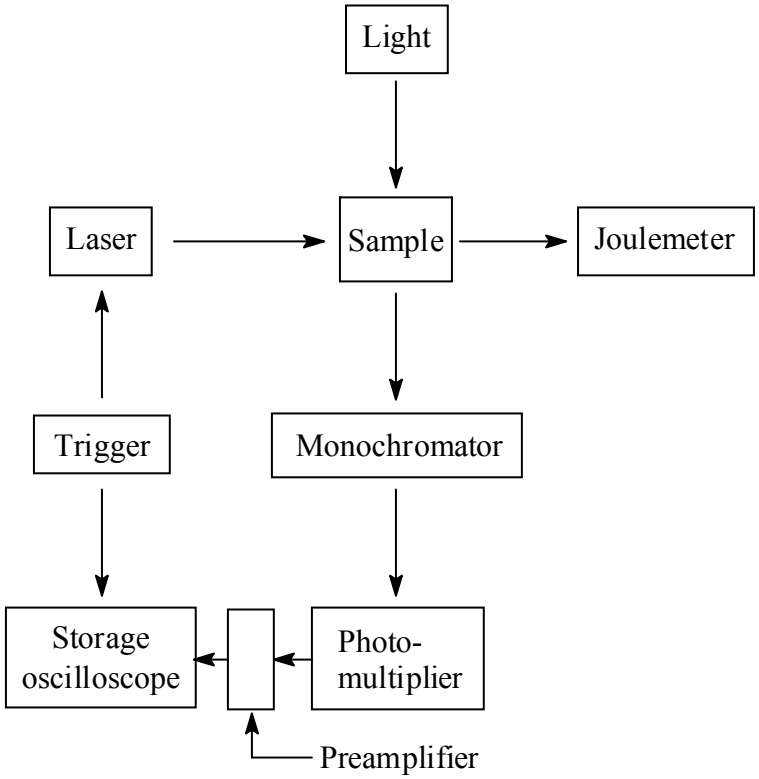


Figure 9: The standard method to observe the photochemical properties of the caged neurotransmitter is depicted in this plot.

Determination of the quantum yield of caged compounds was based on the assumption[85, 86, 94] that the concentration of the transient intermediate is directly proportional to the concentration of compound liberated in the photolysis reaction (resulting of reaction mechanism) and that the absorption of the solution at 308 nm is constant during the measurement (measured by joulemeter). The absorbance of the transient intermediate, A_n, measured at the *n*th laser pulse with mixing of the solution after each pulse, is given[16]:

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

ε_M is the molar extinction coefficient of the transient intermediate, l is the path length of the analysis light, c_0 is the initial concentration of the caged compound, Φ is the quantum yield, n_A is the number of absorbed photons, V the total volume of the solution, n the number laser pulses and F is the fraction of the sample solution irradiated by the laser flash. From the slope of $\ln(A_n)$ versus (n-1) one can obtain Φ (eq 1). From the intercept ε_n can be estimated[18-20, 44-46, 72] (figure 10). The error on ε_n is rather larger because the fraction of sample solution (F) irradiated can only be estimated

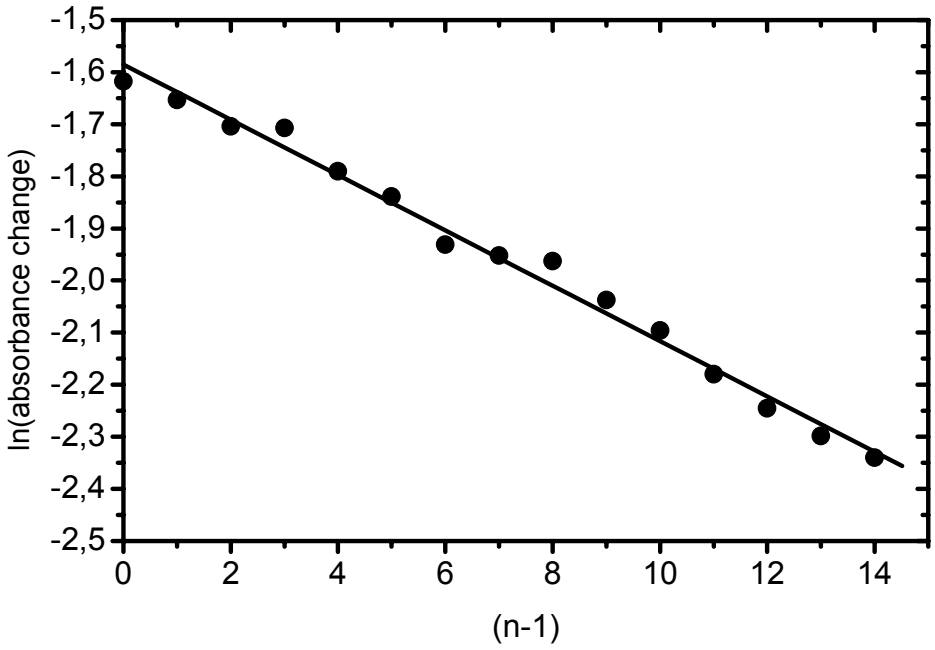


Figure 10: A sample of α,4-DCNB-D-aspartate **32** (500 µM, 100 mM phosphate buffer, pH = 7.0, 50 µl) was irradiated with 15 consecutive laser pulses (10 ns, 308 nm, 10 mJ). The logarithm of the transient absorption immediately after the laser pulse (0.5 µs) was plotted versus the number of the laser pulses – 1. From the slope of the linear fit the quantum yield can be obtained and from the intercept the extinction coefficient of the *aci*-nitro intermediate can be estimated. Each data point represents the mean of three experiments.

Between pH = 5 and pH = 10 the quantum yield is strongly pH-dependent with a maximum between 6 and 7. It can be assumed that this behavior has to be attributed to the protonation of the α-carboxy group in the same manner as for the pH-dependence of the rate constant. Again a distinct change of behavior has been found around pH = 5 (table 3 and figure 11). Obviously, the α-carboxy group in **32** has a higher pK_a than the one in the *aci*-nitro

intermediate, which is in agreement with what one would expect from the resonance structures. (To compare with **31**, see appendix I, table 2 and figure 4)

pH	5	6	7	8	9	10
Φ	0.056	0.13	0.14	0.07	0.06	0.03

Table 3: The values of quantum yield of **32** at various pH values between 5 and 10.

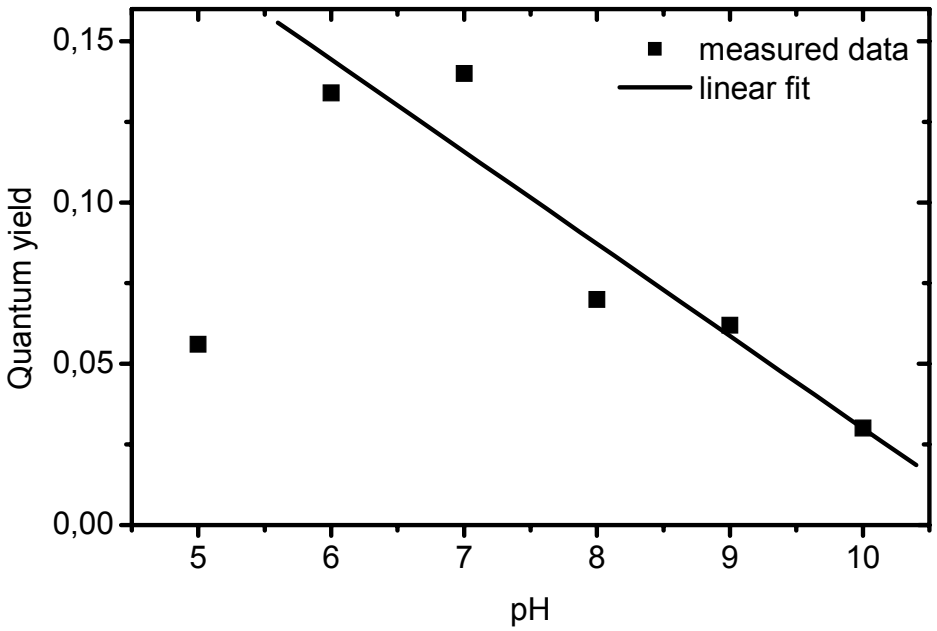


Figure 11: The quantum yield for the photorelease of D-aspartate from α,4-DCNB-D-aspartate **32** (500 μM) is shown. A linear fit (straight line) for pH ≥ 6 is shown. Below pH = 6 the quantum yield becomes smaller again.

The quantum yield for **33** was determined to 0.10, which is just a little bit lower than for the respective α,4-DCNB compound (**32**, 0.14), but still satisfying for the biophysical investigations.

The quantum yields of the synthesized caged compounds are summarized in table 4. The non-water soluble substances (**37** and **38**) are measured in water DMSO (1:1) mixture and water soluble substances are measured in water at pH = 7.

	31	32	33	34	35	36a	37	38
Φ	0.18	0.14	0.10	0.10	0.16	0.18	0.14	0.08

Table 4: Quantum yields of the synthesized caged compounds are shown. The substances **37** and **38** are measured in water/DMSO (1:1), all other in water at pH = 7.

3.3 Kinetic Investigations of Neurotransmitter-Receptors

In kinetic measurements the concentration of neurotransmitter generated by photolysis of a caged compound has to be determined[39]. Before the photolysis experiments a known concentration of free neurotransmitter is applied to the cell and the amplitude of the resulting current is used to calibrate the concentration of neurotransmitter generated in the photolysis experiment. Cell-flow experiments are also used after laser pulse experiments to check if the laser pulses have damaged the cell or the receptors. The cell-flow device is used both to remove the neurotransmitter from the cell culture dish and to add caged neurotransmitter.
The result obtained by laser-pulse photolysis and the single channel current-recording technique[95, 96] can be compared only at low neurotransmitter concentration at which receptor desensitization is relatively slow.

3.3.1 Cell-Flow Technique

Desensitization of neurotransmitter-receptors (the reversible inactivation of receptor by its specific neurotransmitter) was first discovered in studies with the nicotinic acetylcholine receptor and appeared to be slow, occurring in the second time region[97]. When it was discovered, that receptors desensitize in the millisecond time region, a device for flowing solutions containing neurotransmitter over single cells was developed[96, 98-102]. The current due to opening of receptor channels can then be recorded by the whole cell current recording technique. The time resolution of this method is about 10 ms[39]. To obtain a better time resolution, the flow rate of the neurotransmitter solution could be increased. However, if one increases the flow rate of the solutions over the cell, turbulent flow and mixing occurs between the neurotransmitter solution and the buffer solution surrounding the cell. Consequently, the concentration of neurotransmitter in the solution equilibrating with the cell surface receptors is not known.

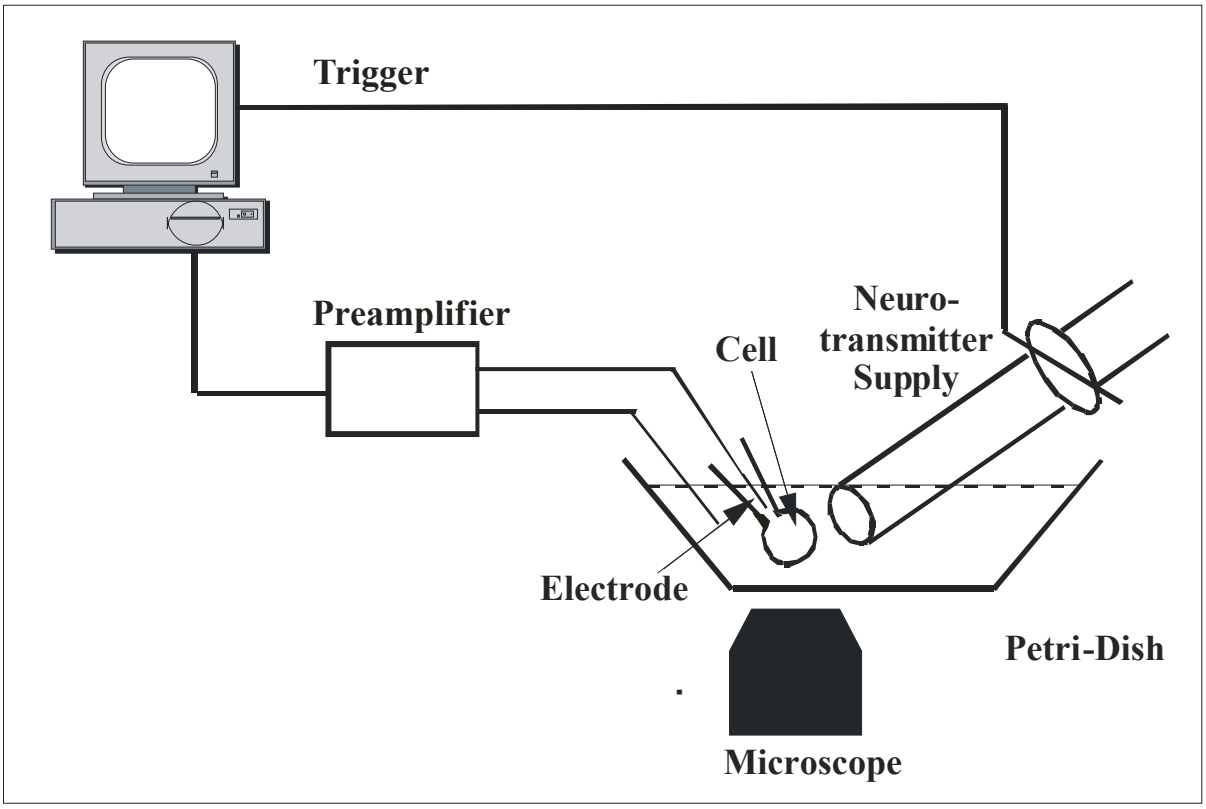


Figure 12: The standard method to observe the biological properties of neurotransmitter is depicted in this plot. By triggering the valve at the port hole of the supply-tube the neurotransmitter is flowed over the cell. The current through the cell membrane is measured by the electrode.

3.3.2 Photolysis Methods

The near-UV 300-360 nm region is optimal for photolysis of 2-nitrobenzyl compounds, which gives the advantages that this class of caged compounds can be readily handled in subdued daylight without significant photolysis. This region also has other advantages; biological compounds are optically transparent in this wavelength range, and the potential photochemical damage to proteins and nucleotides that is anticipated from shorter wavelength irradiation is avoided. An other advantage of avoiding to work in the visible region is that this region is then available for visible absorption and fluorescent spectroscopic analysis following pulse photolysis[103]. Additionally, these methods are fast (the time resolution of a laser pulse photolysis technique is six orders of magnitude better than of cell-flow technique).

A. Flash Lamps: Continuous arc lamps and flash lamps may be used to initiate photolysis of caged compounds. In many situations flash lamps are the optimal light source for photolysis experiments. They are cheaper than lasers and are generally more easily accommodated as a component of an existing apparatus. Their longer pulse times could in principle offer advantages over laser pulses if the lifetimes of excited states of the caged compounds are in microsecond to millisecond range, because multiple excitations of molecules from the ground state and hence more extensive photolysis would then be possible with flash lamps, but flash lamps are not useful, when rapid patterns of stimulation are required. However, the evidence let presume that the excited state of 2-nitrobenzyl compounds have subnanosecond lifetimes[86].

B. Laser-Pulse Photolysis Technique: Advantages of the laser-pulse photolysis technique are time resolution, monochromaticity, and the relative ease of focusing and manipulating the direction and shape of their light beam.

A cell attached to a current-recording electrode is preequilibrated with a biologically inert photolabile precursor of neurotransmitter (caged neurotransmitter). At zero time a pulse of light photolyzes the caged compound in the microsecond time region. The liberated neurotransmitter binds to receptors on the cell surface and initiates the opening of transmembrane channels; the resulting current is measured. The time resolution of the method is governed by the rate with which the neurotransmitter is liberated by a light pulse.

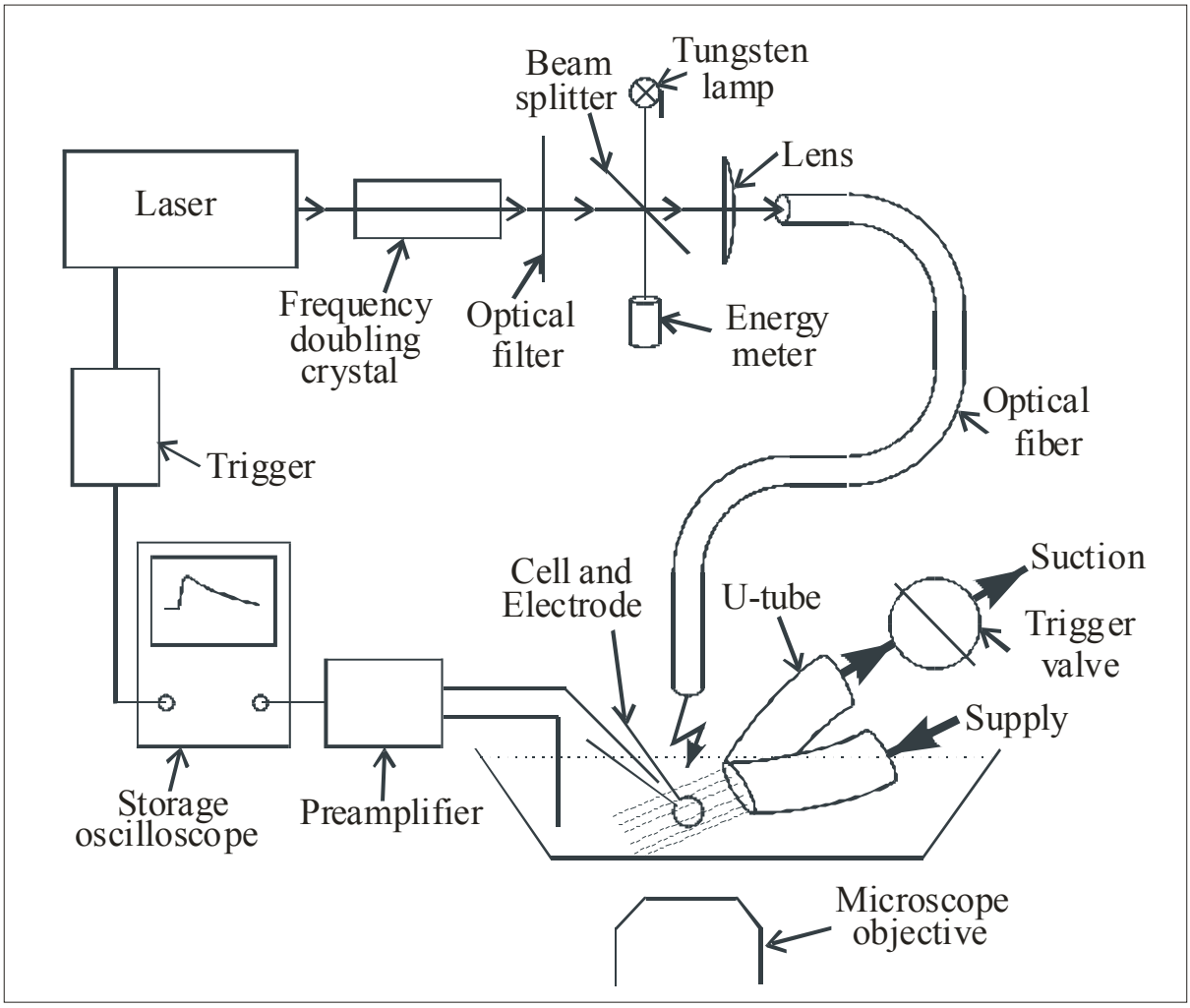


Figure 13: The setup for laser-pulse photolysis, the flow device is used to equilibrate the cell surface with neurotransmitter. The flow is stopped and the neurotransmitter is released upon a laser flash. This allows improving the time resolution compared to flow experiments.

3.3.3 Biological Characterization of Caged Compounds

To obtain a better understanding of the glutamate transport process, it is important to study the molecular reactions of glutamate transporters by time-resolved techniques with time resolution of microseconds to milliseconds. The synthesized caged aspartates (**31** and **32**) are investigated with respect to their photochemical and biological activity. We have used current recording methods using human embryonic kidney (HEK) cells expressing the excitatory amino acid carrier 1 (EAAC1).

As control, we first characterized the steady-state kinetic properties of EAAC1 upon D-aspartate activation. Rapid application of 80 µM D-aspartate evoked a concentration-dependent EAAC1 whole-cell steady-state current (figure 14) in the presence of intracellular rhodanide. Under these conditions mainly the uncoupled anion component ($I^{Glu^-}_{anionic}$) of the current is observed. When L-glutamate was used instead of D-aspartate we observed a small, rapidly decaying transient current component in addition to the steady-state current. The time resolution of the rapid solution exchange method, however, is insufficient to obtain detailed information about this current component[104].

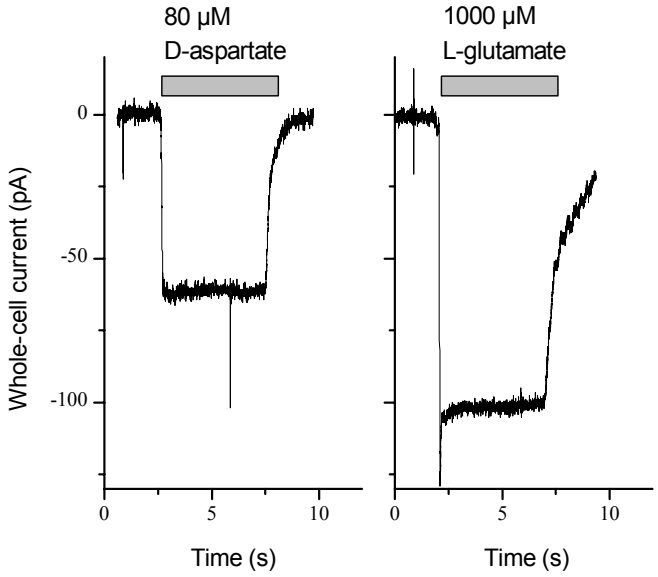


Figure 14: Typical whole-cell current of a EAAC1-expressing HEK 293 cell upon rapid application of 80 µM D-aspartate and 1000 µM L-glutamate (KSCN-based intracellular solution, T = 22 °C, pH = 7.4, V = 0 mV). The application period of the substrates with the rapid solution exchange device is indicated by the bars. Leak currents are subtracted. For n = 5 experiments with 2 cells the ratio of the maximum currents at saturating substrate concentration between D-aspartate and L-glutamate was 0.54 ± 0.04.

When internal SCN^- is replaced by Cl^- the coupled transport current ($I^{Glu^-}_{Na^+/K^+}$) can be measured at V = 0 mV transmembrane potential (Figure 15). This current component ($I^{Glu^-}_{Na^+/K^+}$) is also reduced by a factor of 0.62 ± 0.05 (n = 10, 3 cells) when D-aspartate is

applied to the transporter instead of L-glutamate, in the same range as published previously[105] for the human EAAT3 expressed in *Xenopus* oocytes (0.82). This result indicates that the steady-state transport rate is smaller for D-aspartate than for L-glutamate.

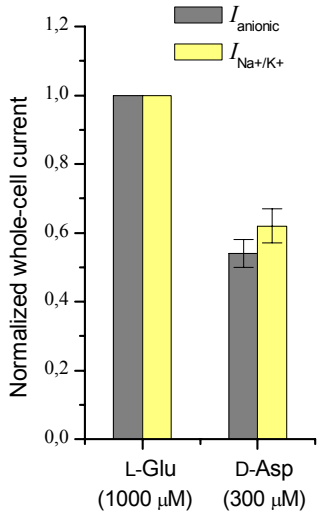


Figure 15: Relative steady-state whole-cell current amplitude induced by 1000 µM L-glutamate (left) and 300 µM D-aspartate (right). The white bars represent the coupled transport current (no electrochemical gradient for anions, KCl-based intracellular solution, n = 6, 3 cells), the grey bars the uncoupled anionic current (KSCN-based internal solution, n = 6, 3 cells). The transmembrane potential was 0 mV.

In second step it was tested whether these compounds inhibit or activate EAAC1. A solution of 100µM free D-aspartate was applied to the cell with a fast solution-exchange device. Afterwards we repeated the experiment with the same cell and coapplied 1 mM of either α-CNB- or α,4-DCNB-D-aspartate together with 100 µM of free neurotransmitter (in a similar experiment we used 500 µM α-CNB-D-aspartate together with 100 µM D-aspartate). The current response of the cell in all experiments was the same within experimental error (figure 16). It was found that all measured substances were inactive before photolysis and should be suitable tools for the investigation of EAAC1. These results demonstrate that caged D-aspartates are biologically inert with respect to glutamate transporters.

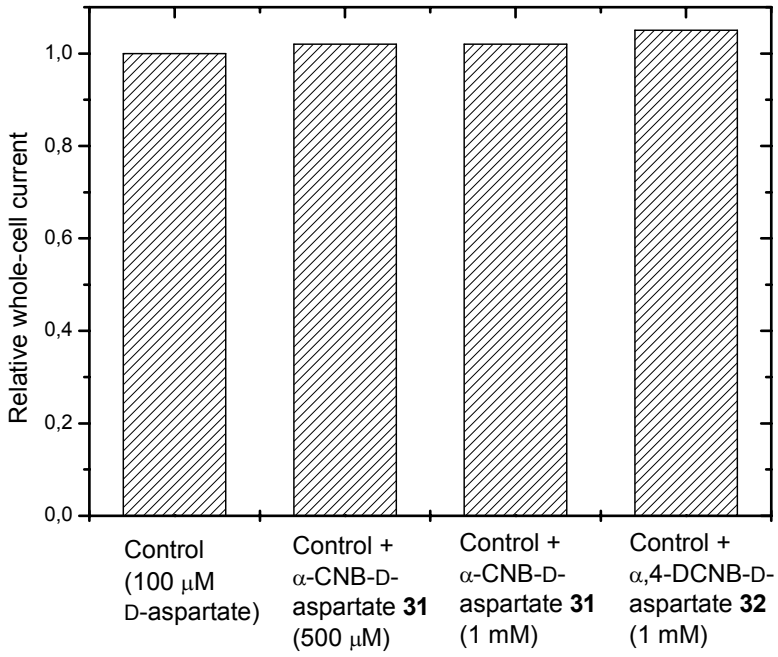


Figure 16: Whole-cell current recording for EAAC1 expressed in HEK 293 cells with 100 μM free aspartate, 100 μM free aspartate in the presence of 500 μM, and 1mM α-CNB-D-aspartate **31**, and 100 μM free aspartate in presence of 1 mM α,4-DCNB-D-aspartate **32**.

In an other experiment we applied 150 μM α,4-DCNB-D-aspartate **32** to the cell with the same rapid solution-exchange device, stopped and then irradiated (345 nm) to release free D-aspartate. Upon release, the current rose with a time constant of 4.3 ms to a new steady-state level (figure 17, for **33** see appendix III, figure 2).

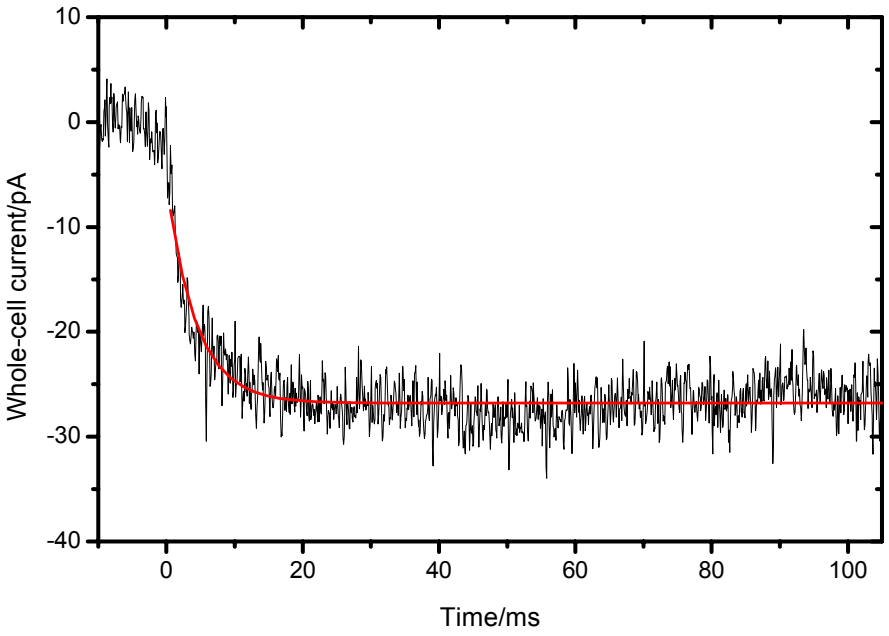


Figure 17: Laser-pulse photolysis experiment with 150 µM α,4-DCNB-D-aspartate (**32**); D-aspartate was released upon application of a 345 nm, 10 ns laser pulse (400 mJ/cm^2); the current was recorded at U = 0 mV; the liberated D-aspartate concentration was estimated to be 15 µM.

In similar experiments we have characterized the biological properties of α,4-DCNB GABA by using current recordings from GABA transporter-expressing HEK 293 cells. GAT-1 was used as a model system because α-CNB-caged GABA inhibits this transporter at high concentrations, as shown in figure 18. However, when the same concentration (1 mM) of α,4-DCNB was coapplied together with 100 µM free GABA, no inhibition of the transport current was observed, indicating that, in contrast to the α-CNB caged compound, α,4-DCNB is not an inhibitor of the GABA transporter.

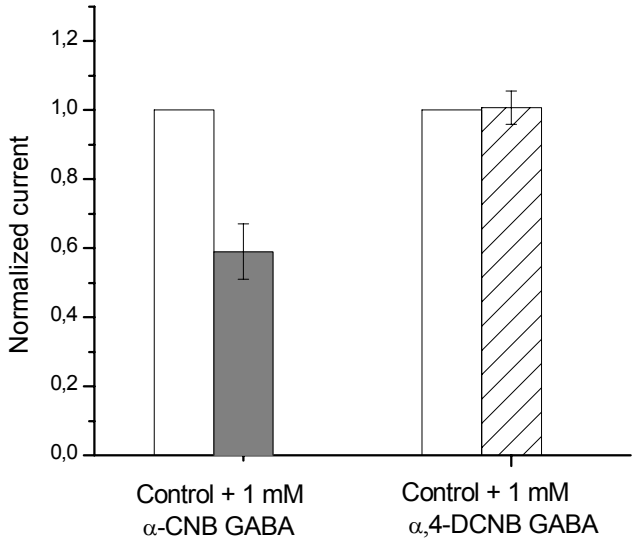


Figure 18: α,4-DCNB GABA does not inhibit the GABA transporter. Normalized current responses recorded when 1 mM α-CNB GABA (grey bar) and 1 mM α,4-DCNB GABA (hatched bar) were coapplied to GAT-1 together with 200 μM and 100 μM GABA, respectively (open bars). The holding potential was –40 mV.

A typical laser pulse photolysis experiment is shown in figure 19B and compared to a rapid solution exchange experiment performed with the same cell with 100 μM free GABA (figure 19A). Clearly, photolytic release of GABA from the caged precursor evokes an inwardly-directed current of the same steady-state amplitude as that evoked by application of 100 μM GABA. This current is not observed when the laser is flashed in the absence of caged GABA from the extracellular solution (figure 19D), demonstrating that it is not caused by a photolysis artifact. In contrast to the solution exchange experiment, several pre-steady-state phases of the current can be resolved, indicating that the new caged GABA will be very useful for future kinetic investigations of the GABA transporter. The same results can not be obtained with α-CNB caged GABA. Due to inhibition the observed current is diminished (figure 19C) compared to α,4-DCNB caged GABA (figure 19D).

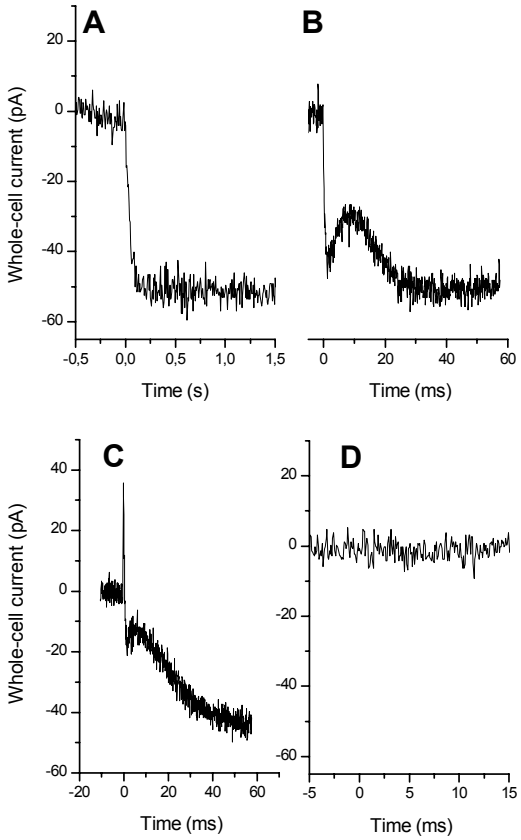
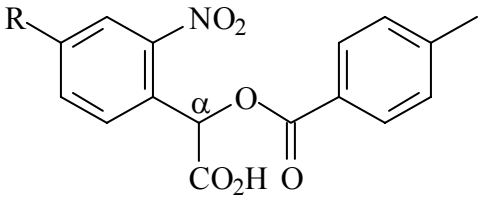


Figure 19: Photolysis of α,4-DCNB GABA evokes currents in GAT-1-transfected cells. (A) Rapid solution exchange experiment with a GAT-1 expressed in HEK 293 cell. 100 µM GABA was applied at time t = 0 s. (B) Laser-pulse photolysis experiment with the same cell in the presence of 1 mM α,4-DCNB GABA. (C) Current evoked by photolysis of 1 mM α-CNB-GABA in the same cell. (D) Laser irradiation of the same cell in the absence of α,4-DCNB GABA does not generate a current response.

One would expect, that the not yet synthesized α,5-DCNB GABA should combine the advantages of the α-CNB GABA (fast photolysis) and the α,4-DCNB GABA (biological inert even at high concentrations).

3.4 Investigation of the Hydrophilicity

The hydrophilicity was investigated by thin layer chromatographies (TLC) with various eluants using silica gel plates. For this investigation we have synthesized two derivatives of *p*-poluic acid (**6**) with α-CNB and α,4-DCNB[106] caging groups. These derivatives exhibit a relatively high hydrophobicity compared to other compounds. This has the advantage, that the effects of an increased hydrophilicity of the caging group can be studied more easily. The results in table 5 show that the introduction of additional carboxy group into aromatic ring increases the hydrophylicity.



R = H **36a**
R = CO_2H **36b**

		R_f **36a**	R_f **36b**
Acetone% 75	Water% 25	0.87	0.83
90	10	0.84	0.70
95	5	0.59	0.23
100	0	0.46	0.13
Acetone% 67	Methanol% 33	0.78	--
80	20	0.65	--

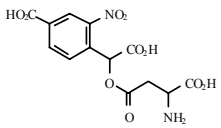
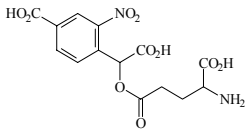
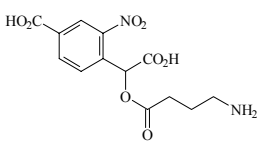
Table 5: The comparison of R_f-values shows clearly that the introduction of additional carboxy group into aromatic ring of the caging group increases the hydrophilicity.

4. Summary

o-Nitrobenzyl protected bioactive compounds (caged compounds) are useful tools in biophysics. Their photo lability allows the controlled release of biologically active substances with high temporal precision.

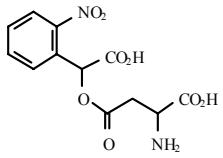
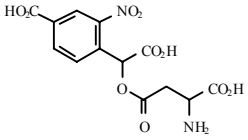
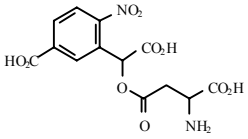
The first goal of this work was the improvement of the hydrophilicity compared to α-CNB, therefore the α,4-DCNB was synthesized. The results of our investigations show as expected, that the hydrophilicity was improved by introduction of an additional carboxy group into the aromatic ring, we have investigated the photochemical and physiological of this compound. However, the synthesis requires more steps. Therefore the improved biological properties have to be achieved by a more costly synthetic procedure.

It is also shown that the caged compound *α*,4-DCNB GABA (**35)** has better biological characterizations than the known α-CNB GABA (some of the photochemical details are shown in appendix II). The caged compound **35** is inactive under physiological conditions and shows no interaction with proteins or membranes.

	t_1/μs	t_2/μs	Φ
32 	110	508	0.14
34 	77	426	0.10
35 	57	211	0.16

The α,4-DCNB caged glutamate (**34**) is synthesized and investigated. (Results of photochemical investigations of this excitatory neurotransmitter are shown in appendix II.)

Our theoretical investigations suggested, that the α,5-DCNB would release the active compound faster than α,4-DCNB. One would expect a similar hydrophilicity for both compounds, therefore α,5-DCNB-D-aspartate **33** was synthesized. This suggestion is proven by our investigations:

	$t_1/\mu s$	$t_2/\mu s$	Φ
31 	29	308	0.18
32 	110	508	0.14
33 	<u>56</u>	<u>240</u>	0.10

The synthesis of caged compounds with an α,6-DCNB caging group was planed as well. However, due to sterical hindrance the substitution of bromine by a substitute was not possible (details of the calculation are shown in appendix IV).

The thiocyanate ion can be used in characterization of EAAC1 (it can replace the chloride ion and pass through the ionic channel). Therefore we were interested in the synthesis of a caged thiocyanate. Since the thiocyanate has a high nucleophilicity and a low steric demand, it was used as test nucleophile in the reaction, where the S_N2 reaction with other nucleophiles failed. We were able to synthesize the designed compounds. However, the photolysis of caged thiocyanate was not successful.

5. Experimental Section

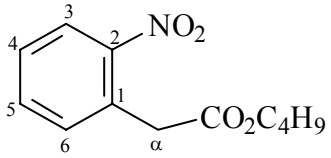
General Remarks

Materials: For column chromatography of the final product Sephadex LH20, BioChemica was used, *N*-(*tert*-butoxycarbonyl)-1-*O*-[*tert*-butyl)-D-aspartic acid and *N*-(*tert*-butoxy carbonyl)-1-*O*-[*tert*-butyl)-L-glutamic acid were obtained from Bachem Biochemica GmbH.

Methods: 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, 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 Elmer Lambda 19. Melting points were determined using a Reichardt Thermovar, Büchi 510.

The ^{13}C-NMR spectra were assigned by increment systems[107] and by comparison with references[108]. ^{1}H-NMR spectra were assigned by increments[107]. IR spectra were assigned by using references[109].

E1: *tert*-Butyl α-(2-nitrophenyl)acetate (4a):



Silver cyanide was prepared by mixing a solution of 2.0 g (31 mmol) sodium cyanide in 50 ml water with a solution of 4.5 g (34 mmol) silver nitrate in 50 ml water at rt. The precipitate was filtered off, washed with water and dried at 0.1 mbar for 10 h. A suspension of 5.0 g (28 mmol) (2-nitrophenyl)acetic acid (**3a**) and 5.2 g (41 mmol) oxalyl chloride in 50 ml dry benzene was stirred for 20 h at rt under nitrogen. The remaining oxalyl chloride was evaporated under reduced pressure at rt. The acid chloride was obtained as clear yellow liquid, which was added to a stirred mixture of 8.0 g (60 mmol) freshly prepared silver cyanide and 3.0 g (41 mmol) *tert*-butanol in 20 ml dry benzene. The mixture was heated to reflux for 8 h and allowed to cool to rt. This mixture was filtered through a silica gel column,

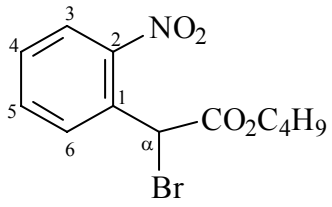
washed with a saturated solution of sodium bicarbonate (3 x 20 ml) and water, dried over sodium sulfate and concentrated to give 6.0 g (25 mmol, 91%) of compound **4a** as a yellow oil.

Anal. for $C_{12}H_{15}NO_4$, Calc.: C, 60.75; H, 6.37; N, 5.90; Found: C, 60.46; H, 6.26; N, 5.99.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.08 (dd, $^3J_{HH}$ = 8.17 Hz, $^4J_{HH}$ = 1.32 Hz, 1 H, Ar-3-H), 7.57 (td, $^3J_{HH}$ = 7.53 Hz, $^4J_{HH}$ = 1.34 Hz, 1 H, Ar-5-H), 7.45 (td, $^3J_{HH}$ = 7.81 Hz, $^4J_{HH}$ = 1.48 Hz, 1 H, Ar-4-H), 7.33 (dd, $^3J_{HH}$ = 7.61 Hz, $^4J_{HH}$ = 1.26 Hz, 1 H, Ar-6-H), 3.93 (s, 2 H, $ArCH_2$), 1.43 (s, 9 H, $C(CH_3)_3$) ppm.

IR (KBr): $\tilde{\nu}$ = 3070 (ν CH aryl), 2980 (ν_{as} CH alkyl), 2931 (ν_s CH alkyl), 1731 (ν C=O), 1613, 1580 (ν and δ ring), 1528 (ν_{as} NO_2), 1451 (ν_s CH_2), 1414, 1393 (δ $C(CH_3)_3$), 1350 (ν_s NO_2), 1153 (ν C–O) cm^{-1}.

E2: *tert*-Butyl α-bromo-α-(2-nitrophenyl)acetate (5a):

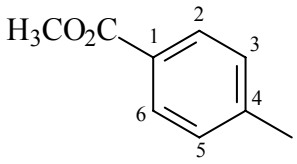


A mixture of 3.0 g (13 mmol) ester **4a**, 2.5 g (14 mmol) *N*-bromosuccinimide (NBS) and catalytic amounts of dibenzoyl peroxide in 25 ml tetrachloromethane was heated to reflux for 72 h. The mixture was allowed to cool to rt, the succinimide and unreacted NBS was filtered off, the filtrate was charged with 1.50 g (8.43 mmol) NBS and catalytic amounts of dibenzoyl peroxide and heated under reflux for further 48 h. The precipitate was filtered off and the filtrate was concentrated under reduced pressure to form a pale yellow oil. The product was separated from the starting material via column chromatography (silica gel, n-hexane/ethyl acetate 3:1) to yield 2.4 g (7.6 mmol, 59%) of **5a** as yellow oil.

Anal. for $C_{12}H_{14}BrNO_4$ Calc.: C, 45.59; H, 4.46; N, 4.43; Found: C, 45.21; H, 4.44; N, 4.35.
^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 7.95 (dd, $^3J_{HH}$ = 8.10, $^4J_{HH}$ = 1.36 Hz, 2 H, Ar-3-H, Ar-6-H), 7.65 (dt, $^3J_{HH}$ = 7.68, $^4J_{HH}$ = 1.33 Hz, 1 H, Ar-5-H), 7.48 (dt, $^3J_{HH}$ = 7.79, $^4J_{HH}$ = 1.40 Hz, 1 H, Ar-4-H), 5.93 (s, 1 H, ArCH), 1.44 (s, 9 H, $C(CH_3)_3$) ppm.

IR (KBr): $\tilde{\nu}$ = 2981 (ν_{as} CH alkyl), 2934 (ν_s CH alkyl), 1736 (ν C=O), 1608, 1578 (ν and δ ring), 1530 (ν_{as} NO_2), 1478 (ν_s CH), 1414, 1394 (δ $C(CH_3)_3$), 1349 (ν_s NO_2), 1140 (ν C–O) cm^{-1}.

E3: Methyl 4-methylbenzoate (7):



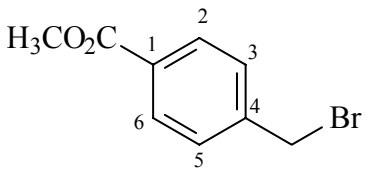
A suspension of 136 g (1.00 mol) **6** and 95.0 ml (1.30 mol) thionylchloride in 190 ml tetrachloromethane was heated to reflux for 16 h. To the clear solution 70 ml methanol was added at 0 °C and the solution was stirred for 4 h at rt. After addition of 30 ml methanol the solution was heated for additional 2 h to reflux. The reaction mixture was treated then with 100 ml ice/water. The organic layer was separated and was extracted with saturated sodium bicarbonate solution (3 x 150 ml) and water (2 x 150 ml). The solution was dried over sodium sulfate and concentrated to give 145 g (967 mmol, 97%) **7** as a light cream-colored solid, mp 35 - 37 °C.

Anal. for $C_9H_{10}O_2$ Calc.: C, 71.98; H, 6.71; Found: C, 71.93; H, 6.92.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 7.92 (d, $^3J_{HH}$ = 8.20 Hz, 2 H, Ar-2-H, Ar-6-H), 7.22 (d, $^3J_{HH}$ = 8.20 Hz, 2 H, Ar-3-H, Ar-5-H), 3.89 (s, 3 H, -OCH_3), 2.39 (s, 3 H, $ArCH_3$) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 167,57 (COOH), 143.93 (Ar-1-C), 129.98 (Ar-2-C & Ar-6-C), 129.46 (Ar-3-C & Ar-5-C), 127.81 (Ar-4-C), 52.31 (OCH_3), 22.01 (Ar-CH_3) ppm,

E4: Methyl 4-(bromomethyl)benzoate (**8**):



To a solution of 70.1 g (467 mmol) **7** in 80 ml benzene was added 24.0 ml (77.7 g, 468 mmol) bromine over a period of 2 h, during which time the solution was irradiated with a 250 W tungsten bulb. After the bromine was added, the mixture was stirred for additional 2 h without irradiation at rt. The solvent was removed from the mixture under reduced pressure and the product was purified by distillation, bp 114 - 119 °C (0.6 mbar), mp 47 - 49 °C, 92.0 g (401 mmol, 86%).

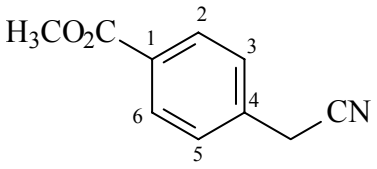
Anal. for $C_9H_9BrO_2$ Calc.: C, 47.19; H, 3.96; Found: C, 47.33; H, 3.95.

^{1}H-NMR (DMSO, 300 MHz) δ = 7.98 - 7.93 (m, A-portion of an $[AB]_2$-system, 2 H, Ar-2-H, Ar-6-H), 7.63 - 7.57 (m, B-portion of an $[AB]_2$-system, 2 H, Ar-3-H, Ar-5-H), 4.77 (s, 2 H, $ArCH_2$), 3.86 (s, 3H, $-OCH_3$) ppm.

^{13}C-NMR (DMSO, 75 MHz) δ = 165.70 (COOH), 143.22 (Ar-4-C), 129.50 ((Ar-2-C & Ar-6-C) or (Ar-3-C & Ar-5-C)), 129.44 ((Ar-2-C & Ar-6-C) or (Ar-3-C & Ar-5-C)), 129.23 (Ar-1-C) 52.12 (OCH_3), 33.04 (Ar-CH_2) ppm.

IR (KBr), $\tilde{\nu}$ = 3422 (2ν C=O), 3018 (ν CH aryl), 2959 (ν_{as} CH_2), 2842 (ν_s CH_2), 1722 (ν C=O), 1682, 1612 (ν and δ ring), 1434 (δ_s CH_2), 1281 (ν C–O), 1110 (ν C–O), 604 (ν C–Br) cm^{-1}.

E5: Methyl 4-(cyanomethyl)benzoate (**9**):



A solution of 45.0 g (691 mmol) potassium cyanide in 70 ml water was added dropwise within 30 min to a solution of 80.0 g (349 mmol) **8** in 350 ml ethanol. The solution was heated under reflux for 3 h and ethanol was removed from the dark violet mixture. After addition of 50 ml water, the mixture was extracted with diethyl ether (3 x 80 ml) and the combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The nitrile **9** was purified through column chromatography on silica gel with n-hexane/ethyl acetate (3:1) yielding 45.3 g (258 mmol, 74%) white solid, mp 57 - 59 °C.

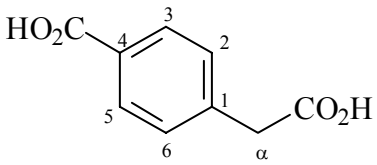
Anal. for $C_{10}H_9NO_2$ Calc.: C, 68.56; H, 5.18; N, 8.00; Found: C, 68.55; H, 5.19; N, 7.93.

^{1}H-NMR (DMSO, 300 MHz): δ = 8.02 - 8.00 (m, A-portion of an $[AB]_2$-system, 2 H, Ar-2-H, Ar-6-H), 7.54 - 7.49 (m, B-portion of an $[AB]_2$-system, 2 H, Ar-3-H, Ar-5-H), 4.19 (s, 2 H, Ar-CH_2), 3.87 (s, 3 H, -OCH_3) ppm.

^{13}C-NMR (DMSO, 75 MHz) δ = 165,75 (COOH), 136.70 (Ar-4-C), 129.50 ((Ar-2-C & Ar-6-C) or (Ar-3-C & Ar-5-C)), 129.44 ((Ar-2-C & Ar-6-C) & (Ar-3-C & Ar-5-C)), 129.23 (Ar-1-C) 52.12 (OCH_3), 33.04 (Ar-CH_2) ppm.

IR (KBr), $\tilde{\nu}$ = 3411 (2ν C=O), 3062, 3014 (ν CH aryl), 2952 (ν_{as} CH_2), 2923 (ν_s CH_2), 2249 (ν C≡N), 1717 (ν C=O), 1674, 1615 (ν and δ ring), 1416 (δ_s CH_2), 1284, 1111 (ν C–O) cm^{-1}.

E6: (4-Carboxyphenyl)acetic acid (**10**):



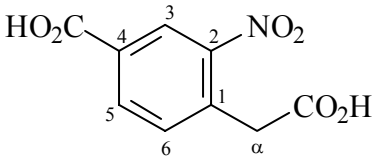
A mixture of 35.0 g (0.200 mol) **9** in 300 ml 45% sulfuric acid was stirred for 1 h at rt and was then heated to reflux for 11 h, during this time 300 ml 45% sulfuric acid was added in several portions. The mixture was stored over night at 5 °C and the residue filtered off. The precipitate was recrystallized from glacial acetic acid yielding 31.2 g (0.173 mol, 87%) **10** as a cream-colored solid which contains three equivalents of water, mp 236 - 238 °C.

^{1}H-NMR (DMSO-d_6, 200 MHz) δ = 7.88 (A-portion of an $[AB]_2$-system, 2 H, Ar-3-H, Ar-5-H), 7.37 (B-portion of an $[AB]_2$-system, 2 H, Ar-2-H, Ar-6-H), 3.66 (s, 2 H, Ar-CH_2), 3.34 (b, H_2O and acid protons) ppm.

^{13}C-NMR (DMSO, 75 MHz) δ = 172.10 (β-C), 167.75 (ArCOOH), 140.05 (Ar-4-C), 129.56 (Ar-3-C, Ar-5-C), 129.17 (Ar-2-C, Ar-6-C), 129.03 (Ar-1-C), 40.44 (α-C) ppm.

IR (KBr): $\tilde{\nu}$ = 3410 (ν OH), 3010 (ν CH aryl), about 3000 (ν OH), 2680 (combination vibration, carboxylic acid), 2560 (combination vibration, carboxylic acid), 1694 (ν C=O), 1426 (δ CH), 1322 (ν C–O), 1293 (ν C–O), 941 (δ OH, oop), 802 (δ CH, oop, 1,4-disubst.) cm^{-1}.

E7: 2-(4-Carboxy-2-nitrophenyl)acetic acid (**3b**):



To 200 ml of a 7:5 mixture of 98% sulfuric acid and fuming nitric acid (100%, d = 1.5 g/ml) 30.0 g (0.167 mol) **10** was added at 0 °C over a period of 8 h. The mixture was then stirred for

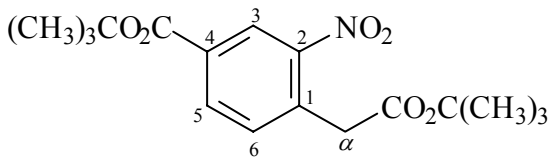
3 h at rt and poured on 600 g ice. The precipitate was washed with cold water, recrystallized from glacial acetic acid and dried under vacuum. The aqueous layers were extracted with ethyl acetate (2 x 100 ml) and the organic layer was concentrated at rt after drying over sodium sulfate to give a combined yield of 26.3 g (0.117 mol, 70%) **3b** as a yellow solid, mp 215 - 217 °C. (lit.[110]: 222 - 223 °C)

^{1}H-NMR (DMSO-d_6, 200 MHz), 8.48 (d, $^{4}J_{HH}$ = 1.7 Hz, 1 H, Ar-3-H), 8.19 (dd, $^{3}J_{HH}$ = 7.9 Hz, $^{4}J_{HH}$ = 1.7 Hz, 1 H, Ar-5-H), 7.69 (d, $^{3}J_{HH}$ = 8.0 Hz, 1 H, Ar-6-H), 4.08 (s, 2 H, Ar-CH_2) ppm.

^{13}C-NMR (DMSO, 75 MHz) δ = 170.75 (AlkCOOH), 165.32 (ArCOOH), 148.48 (Ar-2-C), 134.86 (Ar-1-C), 134.21 (Ar-6-C), 133.69 (Ar-5-C), 131.09 (Ar-4-C), 125.14 (Ar-3-C), 38.87 (α-C) ppm. The signals of the ^{13}C-NMR spectra were assigned using a C/H-COSY.

IR (KBr): $\tilde{\nu}$ = about 3000 (ν OH), 2654, 2555 (combination vibration), 1703 (ν C=O), 1622, 1503 (ν and δ ring), 1534 (ν_{as} NO_2), 1422 (ν_s CH_2), 1348 (ν_s NO_2), 1290 (ν C–O), 1251 (ν C–O) cm^{-1}.

E8: *tert*-Butyl α-[4-(*tert*-butoxycarbonyl)-2-nitrophenyl] acetate (4b):



***A*- With isobutene:** 8.54 g (37.9 mmol) **3b** were suspended in 100 ml chloroform. After addition of isobutene at -78 °C under nitrogen until the volume increased by 5 ml, sulfuric acid (1 ml) was added and the suspension was warmed under stirring to rt in an autoclave. After 10 days it was charged with isobutene again and stirred for an additional week. The unreacted **3b** was filtered off (can be used again) and the chloroform layer was washed with saturated sodium bicarbonate solution (2 x 30 ml) and with water (2 x 30 ml). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 6.51 g (19.3 mmol, 51%) **4b** as a yellow oil.

***B*- With *tert*-butanol:** To a mixture of 10.00 g (44.41 mmol) **3b**, 120 mg (1.00 mmol) 4-dimethylaminopyridine (DMAP) and 20,00 ml (208.0 mmol) *tert*-butanol in 100 ml dichloromethane at 0 °C were added 20.00 g (96.9 mmol) *N*,*N*-dicyclohexylcarbodiimide (DCC). The mixture was stirred first 30 min at 0 °C and then 5 h at rt. The red mixture was filtered and concentrated under reduced pressure. The product mixture was disolved in 100 ml dichloromethane, filtered again and then was washed with 0.5 N hydrochloric acid (2 x 100 ml) and with saturated solution of sodium bicarbonate (2 x 100 ml). The organic solution was dried over sodium sulfate, filtered through a silicagel column and concentrated under reduced pressure. The product was purified through column chromathography (silica gel, *n*-hexane/ethyl acetate 4:1) to yield 8.60 g (25.5 mmol, 57%) **4b** as a yellow oil.

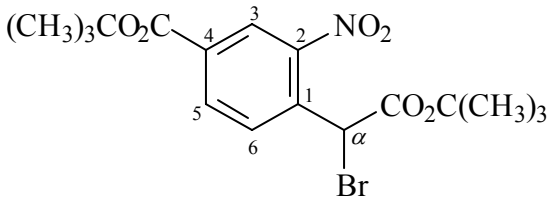
Anal. for $C_{17}H_{23}NO_6$ Calc.: C,60.52; H, 6.87; N, 4.15; Found: C, 60.55; H, 6.85; N, 4.12 .

^{1}H-NMR ($CDCl_3$, 200 MHz) δ = 8.62 (d, $^4J_{HH}$ = 1.7 Hz, 1 H, Ar-3-H), 8.16 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.8, 1 H, Ar-5-H), 7.39 (d, $^3J_{HH}$ = 7.9, 1 H, Ar-6-H), 3.98 (s, 2 H, Ar-CH_2), 1.61 & 1.42 (2 s, 2 * 9 H, -$C(CH_3)_3$) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 168.40 (Alk-COOR), 163.42 (Ar-COOR), 148.87 (Ar-2-C), 134.27 (Ar-1-C), 133.71 (Ar-6-C), 133.32 (Ar-5-C), 132.64 (Ar-4-C), 126.04 (Ar-3-C), 82.48 ($\underline{C}(CH_3)_3$), 82.15 ($\underline{C}(CH_3)_3$), 41.00 (α-C), 28.12 ($C(\underline{C}H_3)_3$), 27.93 ($C(\underline{C}H_3)_3$) ppm.

IR (KBr): $\tilde{\nu}$ = 2980 (ν_{as} CH alkyl), 2934 (ν_s CH alkyl), 1721 (ν C=O), 1623, 1535 (ν and δ ring), 1395, 1381 (δ $C(CH_3)_3$), 1301, 1151 (ν C–O) cm^{-1}.

E9: *tert*-Butyl α-bromo-α-[4-(*tert*-butoxycarbonyl)-2-nitrophenyl]acetate (**5b**):



A solution of 2.00 g (5.93 mmol) **4b**, 1.12 g (6.29 mmol) *N*-bromosuccinimide (NBS) and 0.10 g (0.41 mmol) dibenzoylperoxide in 30 ml tetrachloromethane was heated to reflux for

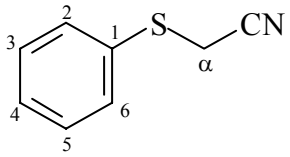
12 h. The mixture was then filtered and the solution was concentrated in vacuum. The product and starting material were separated through column chromatography (silica gel, n-hexane/ethyl acetate 4:1) to yield 1.00 g (2.96 mmol, 50%) unreacted **4b** and 0.85 g (2.04 mmol, 34%) **5b** as a pale brown viscous oil.

Anal. for $C_{17}H_{22}BrNO_6$ Calc.: C, 49.05; H, 5.33; N, 3.36; Found: C, 48.94; H, 5.68; N, 3.02.

^{1}H-NMR ($CDCl_3$, 300 MHz) 8.55 (d, $^4J_{HH}$ = 1.7 Hz, 1 H, Ar-3-H), 8.25 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.7, 1 H, Ar-5-H), 8.05 (d, $^3J_{HH}$ = 8.2 Hz, 1 H, Ar-6-H), 5.96 (s, 1 H, Ar-CH), 1.62 & 1.47 (2 s, 2 * 9 H, -$C(CH_3)_3$) ppm.

IR (KBr): $\tilde{\nu}$ = 2979 (ν_{as} CH), 2934 (ν_s CH), 1723 (ν C=O), 1620, 1538 (ν and δ ring), 1395, 1369 ($C(CH_3)_3$), 1301, 1141 (ν C–O), 628 (ν CBr) cm^{-1}.

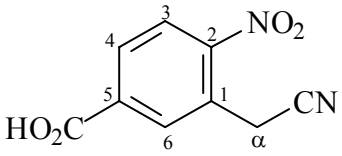
E10: Thiophenoxyacetonitrile (13):[79]



Thiophenol (**11**) (40 ml, 43.12 g, 0.39 mol), α-Chloroacetonitrile (**12**) (30 ml, 35.67 g, 0.47 mol) and sodium carbonate (65 g, 0.61 mol) were mixed in 200 ml acetone, and the mixture was stirred at 50 °C for 5 h. The reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was extracted with 200 ml diethyl ether, washed with water (2 x 50 ml) and dried over sodium sulfate. Evaporation of the solvent in vacuo gave 56 g (0.38 mmol, 97%) pure product.

^{1}H-NMR (DMSO, 500 MHz) δ = 7.54 – 7.51 (m, A portion of a $[AB]_2C$-system, 2H, Ar-2-H, Ar-6-H), 7.39 (m, B portion of a $[AB]_2C$-system, 2H, Ar-3-H, Ar-5-H), 7.30 (m, C portion of a $[AB]_2C$-system, 1H, Ar-4-H), 3.33 (s, 2H, CH_2) ppm.

E11: (5-Carboxy-2-nitrophenyl)acetonitrile (15a):[80]

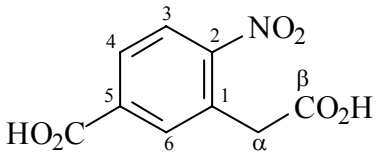


To a stirred suspension of 11.0 g (275 mmol) sodium hydroxide in 100 ml DMSO was added 8.00 g (47.9 mmol) 4-nitrobenzoic acid (**14**) at 18 °C. After 5 min 7.13 g (47.8 mmol) thiophenoxyacetonitrile (**13**) in 100 ml DMSO was added dropwise (over 45 min). The deep blue mixture was stirred for 50 min at 18 °C and then poured into a mixture of 60 ml conc. hydrochloric acid and 500 g ice. The mixture (containing solid particles) was then extracted with ethylacetate (3 x 100 ml), dried over sodium sulfate and concentrated under reduced pressure. The remaining solid was washed with a mixture of chloroform and n-hexane (1:3) to yield 7.70 g (37.3 mmol, 78%) product, mp 183 - 185°C.

^{1}H-NMR (DMSO, 200 MHz) δ = 8.27 (d, $^4J_{HH}$ = 1.6, Hz, 1 H, Ar-6-H), 8.23 (d, $^3J_{HH}$ = 8.5 Hz, 1 H, Ar-3-H), 8.12 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$=1.6 Hz, 1 H, Ar-4-H), 4.41 (s, 2 H, $ArCH_2$) ppm.

IR (KBr) $\tilde{\nu}$ = about 3000 (ν OH), 2552 (combination vibration), 2258 (ν C≡N),1697 (ν C=O), 1616, 1591 (ν and δ ring), 1533 (ν_{as} NO_2), 1435 (ν_s CH_2), 1346 (ν_s NO_2), 1299 (ν C–O), 1250 (ν C–O) cm^{-1}.

E12: 2-(5-Carboxy-2-nitrophenyl)acetic acid (3c):



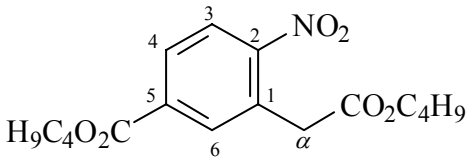
A mixture of 4.50 g (20.8 mmol) nitrile **15a** in 100 ml 20% hydrochloric acid was stirred under reflux for 6 h. The mixture was allowed to cool to rt and was stored over night at 5 °C

and the residue filtered off. The aqueous solution was extracted with ethyl acetate (3 x 50 ml).The organic layer was dried over sodium sulfate and evaporated at rt to give a combined yield of 3.00 g (13.3 mmol, 64%) **3c** as a yellow solid, mp 170 - 171°C.

^{1}H-NMR (DMSO-d_6 200 MHz) δ = 8.30-8.10 (m, 3 H, Ar-protons), 4.44 (s, 2 H, Ar-CH_2) ppm.

IR (film): $\tilde{\nu}$ = 3500-2500 (ν -OH), 1678 (ν C=O), 1615, 1587, (ν and δ ring), 1521 (ν_{as} NO_2), 1400 (δ CH_2), 1348 (ν_s NO_2), 1295, 1124 (ν C–O) cm^{-1}.

E13: *tert*-Butyl α-[5-(*tert*-butoxycarbonyl)-2-nitrophenyl]acetate (4c):[111]



A solution of 3.40 g (15.1 mmol) dicarboxilic acid **3c** and 0.30 ml perchloric acid (70%) in 80.0 ml (594 mmol) *tert*-butylacetate was stirred at rt for 48 h. The clear orange solution was neutralized with saturated sodium bicarbonate solution and extracted with diethyl ether (2 x 50 ml). The combined organic layers were washed with water (2 x 40 ml), dried over sodium sulfate and concentrated under reduced pressure. The remaining oil was dissolved in a n-hexan/diethyl ether mixture (4:1) and filtered through a silica gel column to give 2.95 g (8.74 mmol, 58%) compound **4c** as a pale yellow solid, mp 92 - 94 °C.

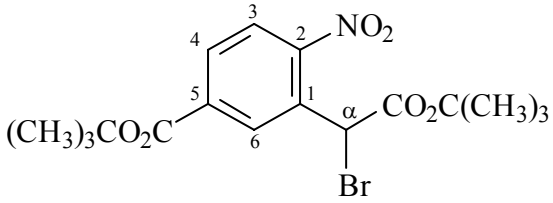
Anal. for $C_{17}H_{23}NO_6$ Calc.: C, 60.52; H, 6.87; N, 4.15; Found: C, 60.31; H, 7.03; N, 4.10.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.10 (d, $^3J_{HH}$ = 8.5 Hz, 1 H, Ar-3-H), 8.03 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H, Ar-4-H), 7.94 (d, $^3J_{HH}$ = 1.8 Hz, 1 H, Ar-6-H), 3.99 (s, 2 H, Ar-CH_2), 1.61 & 1.44 (2 s, 9 H each, *tert*-butyl) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 168.75 (Alk-COOR), 163.62 (Ar-COOR), 151.16 (Ar-2-C), 136.11 (Ar-5-C), 134.27 (Ar-1-C), 130.51 (Ar-3-C), 129.34 (Ar-4-C), 125.08 (Ar-6-C), 82.57, 82.12 ($\underline{C}(CH_3)_3$), 40.89 (α-C), 28.10, 27.96 ($C(\underline{C}H_3)_3$) ppm.

IR (film): $\tilde{\nu}$ = 2979 (ν_{as} CH alkyl), 2930 (ν_s CH alkyl), 1729, 1725 (ν C=O), 1614, 1587 (ν and δ ring), 1526 (ν_{as} NO_2), 1393, 1343 (δ $C(CH_3)_3$), 1298 (ν_s NO_2), 1224, 1153 (ν C–O) cm^{-1}.

E14: *tert*-Butyl α-bromo-α-[5-(*tert*-butoxycarbonyl)-2-nitrophenyl]acetate (5c):



This substance was prepared as previously described for **5b** (**E9**), mp 53 - 55 °C (yield: 53%)

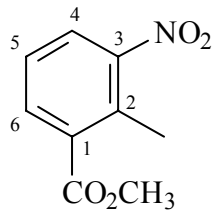
Anal. for $C_{17}H_{22}BrNO_6$: Calc.: C, 49.05; H, 5.33; N, 3.36; Found: C, 49.29; H, 5.01; N, 3.32.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.28 (d, $^4J_{HH}$ = 1.8 Hz, 1 H, Ar-6-H), 8.08 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.80 Hz, 1 H, Ar-4-H), 8.02 (d, $^3J_{HH}$ = 8.5 Hz, 1 H, Ar-3-H), 5.97 (s, 1 H, Ar-CH), 1.65 & 1.43 (2 s, 9 H each, *tert*-butyl) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 164.64 (Alk-COOR), 163.06 (Ar-COOR), 149.87 (Ar-2-C), 136.61 (Ar-5-C), 133.20 (Ar-1-C), 132.85 (Ar-3-C), 130.66 (Ar-4-C), 125.21 (Ar-6-C), 82.83 ($\underline{C}(CH_3)_3$), 65.86 (α-C), 31.60, 28.40, 28.08, 26.92, 22.66, ($C(\underline{C}H_3)_3$) ppm.

IR (film): $\tilde{\nu}$ = 2978, (ν_{as} CH alkyl), 2934 (ν_s CH alkyl), 1721, 1677 (ν C=O), 1608, 1587 (ν and δ ring), 1531 (ν_{as} NO_2), 1456, 1394 (δ $C(CH_3)_3$), 1306 (ν_s NO_2), 1161, (ν C–O) cm^{-1}.

E15: Methyl (2-methyl-3-nitro)benzoate (17):



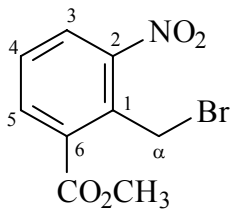
A solution of 1.00 g (5.50 mmol) 2-methyl-3-nitrobenzoic acid (**16**) and 0.5 ml sulfuric acid in 50 ml (39.55 g, 1.23 mol) dry methanol was stirred under reflux for 4 h and then concentrated under reduced pressure. The remaining solid was extracted with 50 ml diethyl ether, washed with 20 ml saturated sodium bicarbonate solution and water (2 x 20 ml) and after drying over sodium sulfate evaporated to yield 1.06 g (5.40 mmol, 98%) **17** as a pale yellow solid, mp 62 - 63 °C.

Anal. for $C_9H_9NO_4$ Calc.: C, 55.39; H, 4.65; N, 7.18; Found: C, 55.32; H, 4.60; N, 7.03.

^{1}H-NMR (DMSO, 200 MHz) δ = 7.98 (dd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-4-H), 7.84 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-6-H), 7.38 (dd, $^3J_{HH}$ = 7.9 Hz, $^3J_{HH}$ = 8.1 Hz, 1 H, Ar-5-H), 3.94 (s, 3 H, OCH_3) 2.62 (s, 3 H, Ar-CH_3) ppm.

IR (film): $\tilde{\nu}$ = 3050 (ν C-H aryl), 2950, (ν CH alkyl), 1721 (ν C=O), 1578, (ν ring), 1521 (ν_{as} NO_2), 1469, 1427 (δ CH_3), 1275 (ν_s NO_2), 1123, (ν C–O) cm^{-1}.

E16: Methyl (2-bromomethyl-3-nitro)benzozate (18):



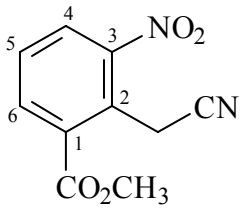
This substance was prepared as described for **5a** (**E2**), mp 57 - 58°C (yield: 99%).

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.02 (dd, $^{3}J_{HH}$= 7.9 Hz, $^{4}J_{HH}$=1.36 Hz, 1 H, Ar-3-H), 7.9 (dd, $^{3}J_{HH}$=8.1 Hz, $^{4}J_{HH}$=1.36 Hz, 1 H, Ar-5-H), 7.46 (dd, $^{3}J_{HH}$=8.0 Hz, $^{3}J_{HH}$=8.1 Hz, 1 H, Ar-4-H), 5.06 (s, 1H, α-CH_2-) 3.91 (s, 3H, OCH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 167.75 (Ar-COOMe), 152.44 (Ar-3-C), 136.63 (Ar-1-C), 135.22 (Ar-4-C), 134.48 (Ar-6-C), 131.06 (Ar-2-C), 128.31 (Ar-5-C), 54.98 (Ar-COO$\underline{C}H_3$), 31.46 (α-CH_2) ppm.

IR (film): $\tilde{\nu}$ = 3078 (ν C-H aryl), 2953, (ν_{as} CH alkyl), 1723 (ν C=O), 1601, 1572 (ν and δ ring), 1529 (ν_{as} NO_2), 1444 (δ CH_2), 1269 (ν_s NO_2), 1120, (ν C–O) cm^{-1}.

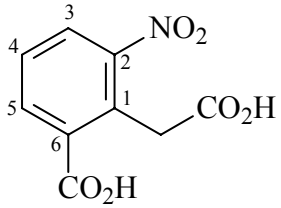
E17: Methyl (2-cyanomethyl-3-nitro)benzoate (19):



A solution of 19.7 g (400 mmol) sodium cyanide in 40 ml water was added dropwise to a solution of 26.0 g (95 mmol) **18** in 80 ml THF. The solution was stirred for 18 h and after addition of 50 ml water, the mixture was extracted with diethyl ether (4 x 80 ml), the combined organic layers was dried over sodium sulfate and concentrated under reduced pressure. **19** was used in the next step without any purification. (18.5 g unpurified material, ca. 84 mmol, ca. 80%)

IR (KBr), $\tilde{\nu}$ = 3093, 3008 (ν CH aryl), 2966 (ν_{as} CH_2), 2950 (ν_s CH_2), 2250 (ν C≡N), 1708 (ν C=O), 1605, 1531 (ν and δ ring), 1435 (δ_s CH_2), 1274, 1128 (ν C–O) cm^{-1}

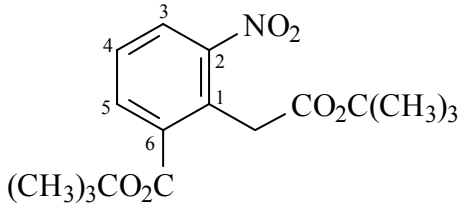
E18: 2-(6-Carboxy-2-nitrophenyl)acetic acid (3d):



A solution of 18.0 g (81.8 mmol) nitrile **19** in 250 ml 20% hydrochloric acid was stirred under reflux for 8 h. The mixture was allowed to cool to rt and was extracted with diethyl ether (3 x 80 ml). The organic layer was dried over sodium sulfate and evaporated to give 14.5 g (64.4 mmol, 68% total yield starting from **18**) **3d** as a yellow solid, mp 150 -152°C (lit.[112]: 154 - 161 °C).

^{1}H-NMR (DMSO, 200 MHz) δ = 8.14 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-3-H), 8.12 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-5-H), 7.63 (dd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 8.2 Hz, 1 H, Ar-4-H), 4.16 (s, 2 H, α-CH_2) ppm.

E19: *tert*-Butyl α-[6-(*tert*-butoxycarbonyl)-2-nitrophenyl]acetate (4d):



This substance was prepared as previously described for **4b** (**E8**), method **A** (with isobutene) (yield: 62%).

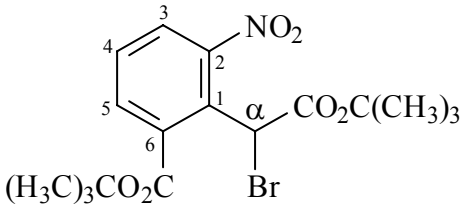
Anal. for $C_{17}H_{23}NO_6$ Calc.: C, 60.52; H, 6.87; N, 4.15; Found: C, 60.88; H, 6.83; N, 3.85.

^{1}H-NMR ($CDCl_3$, 500 MHz) 8.02 (dd, $^3J_{HH}$= 7.9 Hz, $^4J_{HH}$ = 1.40 Hz, 1 H, Ar-3-H), 7.94 (dd, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-5-H), 7.45 (dd, $^3J_{HH}$ = 8.0 Hz, $^3J_{HH}$ = 8.0 Hz, 1 H, Ar-4-H), 4.20 (s, 2 H, α-CH_2), 1.60 & 1.44 (2s, 9 H each, $C(CH_3)_3$) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 168.53 (β-C), 165.12 (Ar-COOR), 150.98 (Ar-2-C), 135.21 (Ar-1-C), 133.89 (Ar-6-C), 129.33 (Ar-5-C), 127.44 (Ar-4-C), 126.83 (Ar-3-C), 82.62 ($\underline{C}(CH_3)_3$), 82.21 ($\underline{C}(CH_3)_3$), 35.30 (α-C), 27.86 ($C(\underline{C}H_3)_3$), 27.26 ($C(\underline{C}H_3)_3$) ppm.

IR (KBr): $\tilde{\nu}$ = 2980 (ν_{as} CH alkyl), 2940 (ν_s CH alkyl), 1732 (ν C=O), 1620, 1534 (ν and δ ring), 1394, 1369 (δ $C(CH_3)_3$), 1287, 1154 (ν C–O) cm^{-1}.

E20: *tert*-Butyl α-bromo-α-[6-(*tert*-butoxycarbonyl)-2-nitrophenyl]acetate (5d):



This substance was prepared as previously described for **5a**, (**E2**) as a yellow oil (yield: 45%).

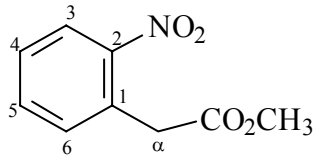
Anal. for $C_{17}H_{22}BrNO_6$ Calc.: C, 49.05; H, 5.33; N, 3.36; Found: C, 48.83; H, 5.42; N, 3.18.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.07 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.5 Hz, 1 H, Ar-3-H), 8.03 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.5 Hz, 1 H, Ar-5-H), 7.54 (dd, $^3J_{HH}$ = $^3J_{HH}$ = 8.0 Hz, 1 H, Ar-4-H), 6.86 (s, 2 H, α-CH), 1.63 & 1.45 (2s, 9 H each, $C(CH_3)_3$) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 165.02 (Alk-COOR), 164.86 (Ar-COOR), 148.98 (Ar-2-C), 134.72 (Ar-1-C), 134.20 (Ar-6-C), 132.32 (Ar-5-C), 129.07 (Ar-4-C), 128.07 (Ar-3-C), 84.10 ($\underline{C}(CH_3)_3$), 83.87 ($\underline{C}(CH_3)_3$), 39.60 (α-C), 28.04 ($C(\underline{C}H_3)_3$), 27.62 ($C(\underline{C}H_3)_3$) ppm.

IR (film): $\tilde{\nu}$ = 3089 (ν Ar-H), 2980 (ν_{as} CH alkyl), 2934 (ν_s CH alkyl), 1750, 1716 (ν C=O), 1605, 1574 (ν and δ ring), 1537 (ν_{as} NO_2), 1426, 1458, 1394 (δ $C(CH_3)_3$), 1287 (ν C-O), 1255 (ν_s NO_2), 1153 (ν C-O) cm^{-1}.

E21: Methyl α-(2-nitrophenyl)acetate (**20a**):



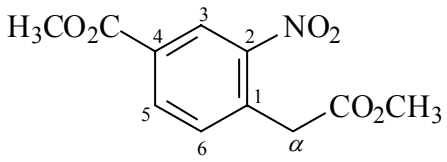
A suspension of 3.20 g (17.7 mmol) α-(2-nitrophenyl)acetic acid (**3a**) and 5.0 ml (7.4 g, 58.2 mmol) oxalyl chloride in 40 ml dry benzene was stirred for 24 h at rt under nitrogen. The remaining oxalylchloride was evaporated under reduced pressure at rt. The yellow solution of acid chloride was then cooled to rt and treated with 8.0 ml dry methanol. The alcoholic mixture was heated to reflux for 8 h and then concentrated under reduced pressure. The remaining liquid was extracted with 50 ml diethyl ether, washed with 20 ml saturated sodium bicarbonate solution and water (2 x 20 ml) and after drying over sodium sulfate evaporated to yield 2.73 g (14.0 mmol, 79%) **20a** as a pale yellow liquid.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.04 (dd, $^{3}J_{HH}$ = 8.2 Hz, $^{4}J_{HH}$ = 1.3 Hz, 1 H, Ar-3-H), 7.57 (ddd, $^{3}J_{HH}$ = $^{3}J_{HH}$ = 7.5 Hz, $^{4}J_{HH}$ =1.3 Hz, 1 H, Ar-5-H), 7.43 (ddd, $^{3}J_{HH}$ = 8.1 Hz, $^{3}J_{HH}$ = 7.5 Hz, $^{4}J_{HH}$ = 1.5 Hz, 1 H, Ar-4-H), 7.36 (dd, $^{3}J_{HH}$ = 7.6 Hz, $^{4}J_{HH}$ = 1.5 Hz, 1 H, Ar-6-H), 4.10 (s, 2 H, α-H) 3.66 (s, 3H, Alk-CO-OCH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 170.36 (Alk-CO-OCH_3), 148.75 (Ar-2-C), 133.66 (Ar-1-C or Ar-3-C), 133.51 (Ar-1-C or Ar-3-C), 130.23 (Ar-5-C), 128.55 (Ar-6-C), 125.48 (Ar-4-C), 51.98 (CO-O<u>C</u>H_3), 39.65 (α-C) ppm.

IR (film): $\tilde{\nu}$ = 3006 (ν C-H aryl), 2955, (ν CH alkyl), 1742 (ν C=O), 1613, 1579, (ν ring), 1527 (ν_{as} NO_2), 1436, 1415 (δ CH_3), 1350 (ν_s NO_2), 1170, (ν C–O) cm^{-1}.

E22: Methyl α-[4-(methyloxycarbonyl)-2-nitrophenyl]acetate (**20b**):



A suspension of 5.96 g (26.5 mmol) **3b** and 5.00 ml (68.5 mmol) thionylchloride in 30 ml tetrachloromethane was heated to reflux for 24 h. To the clear solution 10 ml (7.91 g, 0.25 mol) methanol was added at 0 °C and the solution was stirred for 4 h at rt and heated for additional 2 h to reflux. The reaction mixture was treated then with water. The organic layer was separated and extracted with saturated sodium bicarbonate solution (2 x 15 ml) and water (2 x 15 ml). The solution was dried over sodium sulfate and concentrated to give 4.74 g (18.7 mmol, 70%) cream-colored solid, mp 76 - 77 °C.

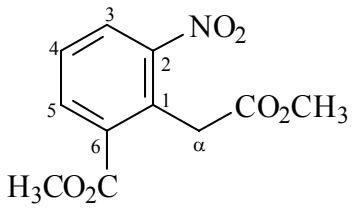
Anal. for $C_{11}H_{11}NO_6$ Calc.: C, 52.18; H, 4.38; N, 5.53; Found: C, 52.24; H, 4.41; N, 5.47 .

^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 8.74 (d, $^4J_{HH}$ = 1.7 Hz, 1 H, Ar-3-H), 8.24 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ =1.7, 1 H, Ar-5-H), 7.37 (d, $^3J_{HH}$ = 8.0 Hz, 1 H, Ar-6-H), 4.10 (s, 2 H, α-H) 3.98 (s, 3H, Ar–CO–OCH_3) 3.72 (s, 3 H, Alk-CO–OCH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 168.61 (Alk-COOMe), 163.63 (Ar-COOMe), 147.70 (Ar-2-C), 133.07 (Ar-1-C), 132.88 (Ar-5-C or Ar-6-C), 132.59 (Ar-5-C or Ar-6-C), 129.92 (Ar-4-C), 125.23 (Ar-3-C), 51.66 (Ar-CO-O<u>C</u>H_3), 51.31 (Alk-CO-O<u>C</u>H_3), 38.43 (α-C) ppm.

IR (KBr): $\tilde{\nu}$ = 3009 (ν C-H aryl), 2956 (ν_{as} CH_2), 2848 (ν_s CH_2), 1732 (ν C=O), 1624, 1498 (ν and δ ring), 1537 (ν_{as} NO_2), 1353 (ν_s NO_2), 1296, 1120 (ν C–O) cm^{-1}.

E23: Methyl α-[6-(methyloxycarbonyl)-2-nitrophenyl]acetate (20c):



This substance was prepared as previously described for **20a** in **E21** (yield: 62% as a white solid), mp 52 - 53 °C (lit.[112]: 51 - 52 °C).

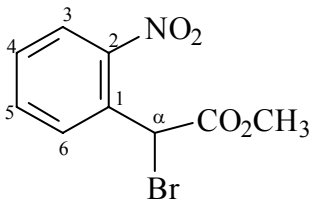
Anal. for $C_{11}H_{11}NO_6$ Calc.: C, 52.18; H, 4.38; N, 5.53; Found: C, 51.91; H, 4.20; N, 5.53.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.18 (d, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-3-H), 8.03 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ =1.4, 1 H, Ar-5-H), 7.37 (d, $^3J_{HH}$ = $^3J_{HH}$ =8.0 Hz, 1 H, Ar-4-H), 4.29 (s, 2 H, α-H) 3.92 (s, 3 H, Ar–CO–OCH_3) 3.73 (s, 3 H, Alk-CO–OCH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 170.24 (Alk-CO-OCH_3), 166.23 (Ar-CO-OCH_3), 151.45 (Ar-2-C), 134.79 (Ar-6-C), 132.97 (Ar-1-C), 129.99 (Ar-3-C), 128.17 (Ar-5-C), 127.89 (Ar-4-C), 52.83 (Ar-CO-OCH_3), 52.28 (Alk-CO-OCH_3), 34.45 (α-C) ppm.

IR (film): $\tilde{\nu}$ = 3015 (ν C-H aryl), 2956 (ν_{as} CH_2), 1730 (ν C=O), 1608, 1577 (ν and δ ring), 1534 (ν_{as} NO_2), 1353 (ν_s NO_2), 1273, 1124 (ν C–O) cm^{-1}.

E24: Methyl α-bromo-α-(2-nitrophenyl)acetate (21a):



This substance was prepared as previously described for **5a** in **E2** (yield: 60%), mp 63 °C (lit.[113]: 61-62 °C)

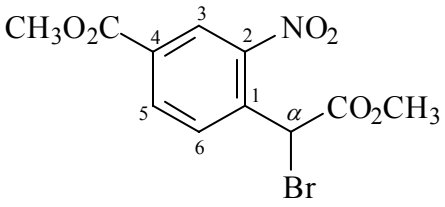
Anal. for $C_9H_8BrNO_4$ Calc.: C, 39.44; H, 2.94; N, 5.11; Found: C, 39.45; H, 2.95; N, 4.96.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.03 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.3 Hz, 1 H, Ar-3-H), 8.00 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-6-H), 7.71 (ddd, $^3J_{HH}$ = $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-5-H), 7.54 (ddd, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.34 Hz, 1 H, Ar-4-H), 6.10 (s, 1 H, α-H) 3.83 (s, 3 H, Ar–CO–OCH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 168.17 (Alk-CO-OCH_3), 147.57 (Ar-2-C), 133.93 (Ar-1-C), 133.11 (Ar-6-C), 130.61 (Ar-4-C or Ar-5-C), 130.01 (Ar-4-C or Ar-5-C), 125.50 (Ar-3-C), 53.69 (CO-OCH_3), 42.29 (α-C) ppm.

IR (film): $\tilde{\nu}$ = 3050 (ν C-H aryl), 2957, (ν_{as} CH alkyl), 1741 (ν C=O), 1519 (ν_{as} NO_2), 1436 (δ CH), 1222 (ν_s NO_2), 1145, (ν C–O) cm^{-1}.

E25: Methyl α-bromo-α-(4-methoxycarbonyl-2-nitrophenyl)acetate (21b):



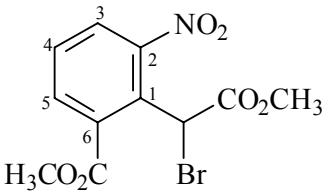
This substance was prepared as previously described for **5a** in **E2** (yield: 42%)

Anal. for $C_{10}H_{10}BrNO_4$ Calc.: C, 39.78; H, 3.03; N, 4.22; Found: C, 40.04; H, 3.00; N, 4.18.

^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 8.64 (d, $^4J_{HH}$ = 1.7 Hz, 1 H, Ar-3-H), 8.32 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.8, 1 H, Ar-5-H), 7.37 (d, $^3J_{HH}$ = 8.2 Hz, 1 H, Ar-6-H), 6.11 (s, 1 H, α-H), 3.99 (s, 3 H, Ar–CO–OCH_3) 3.84 (s, 3 H, β-CO–OCH_3) ppm.

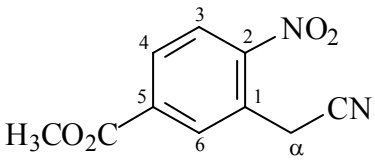
IR (film): $\tilde{\nu}$ = 3008 (ν C-H aryl), 2956 (ν_{as} CH_2), 2847 (ν_s CH_2), 1732 (ν C=O), 1621, 1493 (ν and δ ring), 1538 (ν_{as} NO_2), 1352 (ν_s NO_2), 1302, 1120 (ν C–O) cm^{-1}.

E26: Methyl α-bromo-α-(6-methoxycarbonyl-2-nitrophenyl)acetate (21c):



This substance was prepared as previously described for **5a** in **E2**. The R_f values of the mixture (start material and product) are nearly the same (n-hexane/diethyl ether 1:1); therefore the mixture was used in the next step without any purification.

E27: [5-(Methoxycarbonyl)-2-nitrophenyl]acetonitrile (15b):



A mixture of 2.00 g (9.70 mmol) (5-carboxy-2-nitrophenyl)acetonitrile (**15a**) and 2.00 ml (2.96 g, 23.3 mmol) oxalyl chloride in 50 ml dry benzene was stirred at rt for 48 h under nitrogen. The remaining oxalyl chloride was evaporated under reduced pressure at rt. Methanol (10 ml, 0.25 mol) was added to the cooled solution and the mixture was stirred at 60 °C. After 2 h the reaction mixture was allowed to cool to rt and was poured at 0 °C into a solution of saturated sodium bicarbonate in water (200 ml). The layers were separated and the aqueous layer was washed with diethyl ether (2 x 50 ml). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure to yield 1.97 g (8.90 mmol, 92%) product as a pale yellow solid, mp 112 - 114 °C.

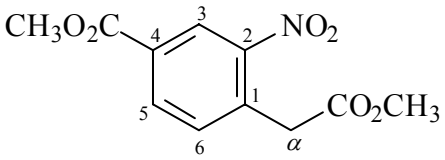
Anal. for $C_{10}H_8N_2O_4$ Calc.: C, 54.55; H, 3.66; N, 12.72; Found: C, 54.25; H, 3.79; N, 12.47.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 8.37 (dd, $^4J_{HH}$ = 1.6 Hz, $^5J_{HH}$ = 0.6 Hz, 1 H, Ar-6-H), 8.23 (dd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.6 Hz, 1 H, Ar-3-H), 8.21 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.6 Hz, 1 H, Ar-4-H), 4.22 (s, 2 H, Ar-CH_2), 4.01 (s, 3 H, O-CH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 164.78 (ArCOOR), 150.37 (Ar-2-C), 135.70 (Ar-5-C), 132.74 (Ar-1-C), 131.23 (Ar-3-C), 126.36 (Ar-6-C), 116.12 (Ar-4-C), 77.95 (CN), 53.50 (O-CH_3), 23.01 (α-C) ppm.

IR (film): $\tilde{\nu}$ = 3122, 3052 (ν C-H aryl), 2958 (ν_{as} CH_2), 2249 (ν C≡N), 1714 (ν C=O), 1614, 1585 (ν and δ ring), 1531 (ν_{as} NO_2), 1428 (ν_s CH_2), 1345 (ν_s NO_2), 1295 (ν C–O), 1270 (ν C–O) cm^{-1}.

E28: Methyl 2-[5-(methoxycarbonyl)-2-nitrophenyl]acetate (**20d**):



A solution of 1.00 g (4.44 mmol) acid **3c** and 0.5 ml sulfuric acid in 50 ml dry methanol was stirred under reflux for 4 h and concentrated under reduced pressure. The remaining solid was extracted with 50 ml diethyl ether, washed with 20 ml saturated sodium bicarbonate solution and water (2 x 20 ml) and after drying over sodium sulfate evaporated to yield 1.00 g (3.95 mmol, 89%) **20c** as a pale yellow solid, mp 63 - 65 °C (lit.[114]: 69 °C).

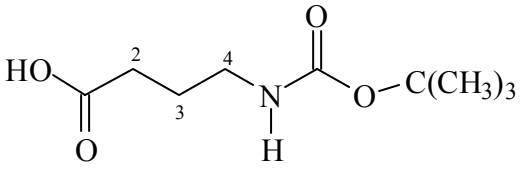
Anal. for $C_{11}H_{11}NO_6$ Calc.: C, 52.18; H, 4.38; N, 5.53; Found: C, 52.10; H, 4.40; N, 5.61 .

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.14 (dd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.6 Hz, 1 H, Ar-3-H), 8.11 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.7 Hz, 1 H, Ar-4-H), 8.03 (dd, $^4J_{HH}$ = 1.7 Hz, $^5J_{HH}$ = 0.6 Hz, 1 H, Ar-6-H), 4.07 (s, 2 H, Ar-CH_2), 3.96 (s, 3 H, Ar-$COOCH_3$), 3.71 (s, 3 H, Alk-$COOCH_3$) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 170.32 (Alk-COOMe), 165.33 (Ar-COOMe), 151.72 (Ar-2-C), 134.95 (Ar-5-C), 134.72 (Ar-3-C), 130.25 (Ar-6-C), 130.14 (Ar-1-C), 125.75 (Ar-4-C), 53.22 (Alk-COO<u>C</u>H_3), 52.80 (Ar-COO<u>C</u>H_3), 39.67 (α-CH_2) ppm.

IR (film): $\tilde{\nu}$ = 3431 (2ν C=O), 3117, 3089, 3053 (ν C-H aryl), 2951 (ν_{as} CH_2), 2880 (ν_s CH_2), 1741, 1722 (ν C=O), 1616, 1587 (ν and δ ring), 1525 (ν_{as} NO_2), 1356 (ν_s NO_2), 1277, 1124 (ν C–O) cm^{-1}.

E29: *N*-(*tert*-butoxycarbonyl)-γ-aminobutyric acid (24)[81]:

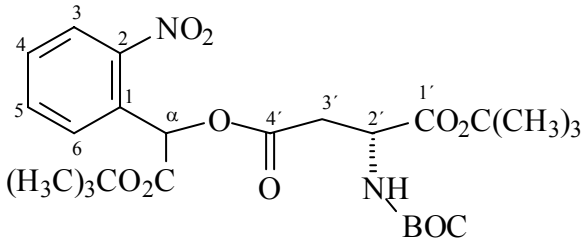


To a solution of 5.00 g (48.0 mmol) 4-aminobutyric acid (GABA, **22**) and 13.5 g (98.0 mmol) potassium carbonate in 60 ml water and 120 ml dioxane at 0 °C was added 11.6 g (53.0 mmol) di-*tert*-butoxy-orthocarbonate (di-BOC, **23**). The mixture was stirred for 4 h at rt and was concentrated under reduced pressure. The residue was dissolved in 100 ml water and washed with ethyl acetate (2 x 50 ml). The mixture was acidified with hydrochloric acid to pH ≈ 3, extracted with ethyl acetate (3 x 100 ml) and dried over sodium sulfate. The solvent was then removed under reduced pressure to give 9.70 g (47.7 mmol, 99%) **24** as a colorless oil.

^{1}H-NMR (DMSO, 200 MHz) δ = 6.81 (t, $^3J_{HH}$ = 5.8 Hz, 1 H, N-H), 3.8-3.2 (broad, 2 H, H_2O), 2.93 (qua, $^3J_{HH}$ = 6.1 Hz, 2H, 4-H), 2.20 (t, $^3J_{HH}$ = 7.4 Hz, 2H, 2-H), 1.60 (qui, $^3J_{HH}$ = 7.3 Hz, 2H, 3-H) 1.38 (s, 9H, *tert*-Butyl) ppm.

IR (film) $\tilde{\nu}$ = 3350 (ν N-H), 2978 (ν_{as} C-H), 2935 (ν_s C-H), 1714 (ν C=O), 1454, 1434 (δ CH_2), 1253 (ν C–N), 1170 (ν C–O) cm^{-1}.

E30: ***N*-(*tert*-Butoxycarbonyl)-4-*O*-[α-(*tert*-butoxycarbonyl)-2-nitrobenzyl]-1-*O*-*tert*-butyl-*R*-aspartic acid (25)**:

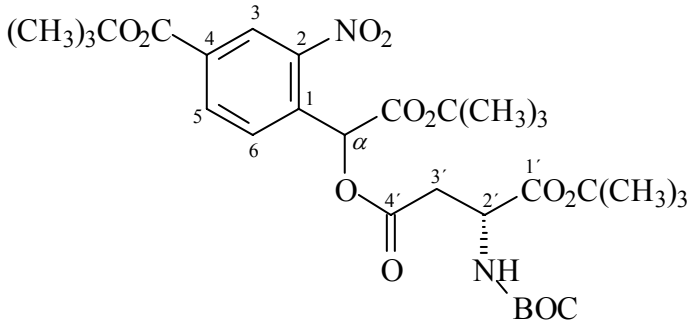


A mixture of 0.24 g (0.83 mmol) *N*-(*tert*-butoxycarbonyl)-1-*O*-*tert*-butyl-*R*-aspartic acid, 0.36 g (1.1 mmol) *tert*-butyl α-bromo-α-(2-nitrophenyl)acetate **5a**, and 0.24 g (1.6 mmol) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 30 ml dry benzene was heated under reflux for 5 h. The mixture was allowed to cool to rt and 20 ml water were added to dissolve the solids. Ethyl acetate (20 ml) was added, the organic layer was removed and the aqueous layer was washed two times with ethyl acetate (20 ml). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure to yield a pale brown oil. The product was purified through column chromatography (silicagel, n-hexane/ethyl acetate 3:1). 0.37 g (0.71 mmol, 85%) 1:1 mixture of diastereomers (estimated from the ^{1}H-NMR integrals of the benzylic proton) of **25** was obtained as yellow oil.

Anal. for $C_{25}H_{36}N_2O_{10}$ Calc.: C, 57.24; H, 6.92; N, 5.34; Found: C, 57.43; H, 6.96; N, 5.08.

^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 8.03 (dd, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 1.1 Hz, 1 H, Ar-3-H), 7.7 – 7.5 (m, 3 H, Ar-4-H, Ar-5-H, Ar-6-H), 6.81, 6.78 (2 s, 1 H, ArCH), 5.64, 5.56 (2 d, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 8.5 Hz, 1 H, NH), 4.53 – 4.47 (m, 1 H, NH-C<u>H</u>), 3.3 – 2.9 (m, 2 H, CH_2), 1.45, 1.44, 1.43, 1.41, 1.40 (5 s, 27H, $C(CH_3)_3$) ppm.

E31: 4-*O*-[α,4-bis-(*tert*-butoxycarbonyl)-2-nitrobenzyl]-*N*-(*tert*-butoxycarbonyl)-1-*O*-*tert*-butyl-*R*-aspartic acid (26):



A mixture of 150 mg (0.520 mmol) *N*-(*tert*-butoxycarbonyl)-1-*O*-[*tert*-butyl)-*R*-aspartic acid, 250 mg (0.600 mmol) *tert*-butyl α-bromo-α-(4-*tert*-butoxycarbonyl-2-nitrophenyl)acetate (**5b**) and 100 mg (0.650 mmol) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 15 ml dry benzene was heated under reflux for 5 h. The mixture was allowed to cool to rt and 20 ml water and 20 ml ethyl acetate were added. The layers were separated and the aqueous layer was extracted with ethyl acetate (2 x 10 ml). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The product **26** was purified by column chromatography (silica gel, n-hexane/ethyl acetate 4:1) yielding 190 mg (0.300 mmol, 58%) of 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.

Anal. for $C_{30}H_{44}N_2O_{12}$ Calc.: C, 57.68; H, 7.10; N, 4.48; Found: C, 57.59; H, 7.31; N, 4.02.

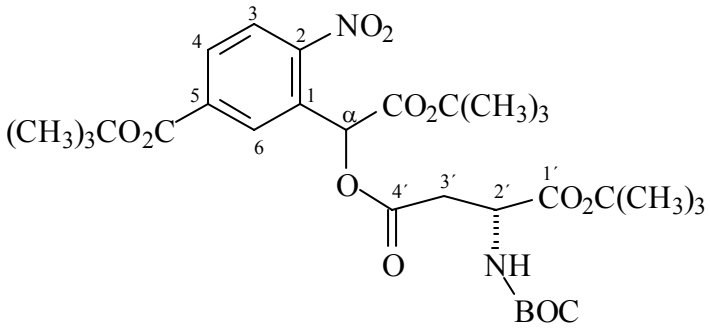
^{1}H-NMR ($CDCl_3$ 300 MHz) δ = 8.56 & 8.57 (2 d, $^{4}J_{HH}$ = 1.4 Hz , $^{4}J_{HH}$ = 1.4 Hz, 1 H, Ar-3-H), 8.23 & 8.22 (2 dd, $^{3}J_{HH}$ = 8.2 Hz, $^{3}J_{HH}$ = 8.2 Hz , $^{4}J_{HH}$ = 1.8 and 1.8 Hz, 1 H, Ar-5-H), 7.72 (d, $^{3}J_{HH}$ = 8.2 Hz, 1 H, Ar-6-H), 6.85 & 6.83 (2s (Integration: 1.3:1), 1 H, α-H), 5.57 & 5.52 (2 d, $^{3}J_{HH}$ = 8.4 Hz, $^{3}J_{HH}$ = 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, (2 s, 9 H, N–CO–C$(CH_3)_3$) 1.45 & 1.44 (2 s, 9 H, Ar–CO–C$(CH_3)_3$), 1.40, 1.39, 1.38 (3 s, 18 H, Alk–CO–C$(CH_3)_3$) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 169.59 & 169.38 (4'-C, 1'-C), 165.53 & 165.45 (β-C), 163.02 (Ar-COO-*tert*-Butyl), 155.48 & 155.41 (NH–CO–O), 148.09 & 148.03 (Ar-2-C), 133.72 (Ar-5-C), 133.201 & 132.935 (Ar-1-C and Ar-4-C), 129.05 & 128.98 (Ar-6-C), 125.90 (Ar-3-C), 84.12 (Ar—CO–O<u>C</u>$(CH_3)_3$), 82.85 & 82.61 & 82.58 (β-C–O<u>C</u>$(CH_3)_3$ and

1‘-C–O<u>C</u>$(CH_3)_3$), 79.93 (NH-CO-O<u>C</u>$(CH_3)_3$), 70.35 & 70.23 (α-C), 50.43 & 50.31 (3‘-C), 36.78 & 36.73 (2‘-C), 28.31 (NH-CO-OC(<u>C</u>$H_3)_3$), 28.09 (Ar-CO-OC(<u>C</u>$H_3)_3$), 27.80 & 27.71 & 27.68 ((Alk-CO-OC(<u>C</u>$H_3)_3$ and 1‘-CO-OC(<u>C</u>$H_3)_3$) ppm.

IR (film): $\tilde{\nu}$ = 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}.

E32: 4-*O*-[*a*,5-bis-(*tert*-butoxycarbonyl)-2-nitrobenzyl]-*N*-(*tert*-butoxycarbonyl)-1-*O*-*tert*-butyl -*R*-aspartic acid (27):

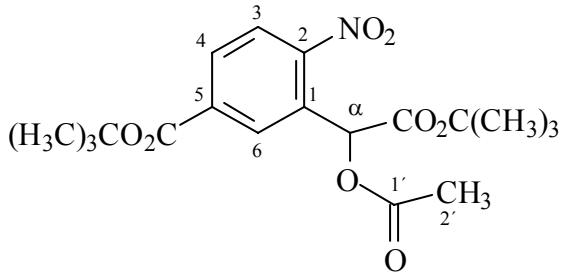


This substance was prepared with 55% yield as previously described for **26** (**E31**). A roughly 1:1.1 mixture of diastereomers (estimated from the ^{1}H-NMR integrals of the benzylic proton) as a pale brown viscous oil.

^{1}H-NMR ($CDCl_3$ 200 MHz) δ = 8.27 & 8.23 (2 d, $^4J_{HH}$ = 1.8 Hz , $^4J_{HH}$ = 1.7 Hz, 1H, Ar-6-H), 8.13 & 8.12 (2 dd, $^3J_{HH}$ = 8.5 Hz, $^3J_{HH}$ = 8.5 Hz , $^4J_{HH}$ = 1.8 and $^4J_{HH}$ = 1.7 Hz, 1H, Ar-4-H), 8.05 & 8.02 (2 d, $^3J_{HH}$ = 8.5 Hz, 1H, Ar-3-H), 6.64 & 6.63 (2s (Integration: 1:1.1), 1H, α-H), 5.52 & 5.45 (2 d, $^3J_{HH}$ = 7.0 Hz, $^3J_{HH}$ = 7.0 Hz, 1H, N-H), 4.60 - 4.40 (m, 1H, 2’-CH), 3.18-2.74 (m, 2H, 3’-H), 1.60 & 1.59 (2 s, 9 H, BOC) 1.44, 1.43, 1.42, 1,393, 1.386 & 1.38, (6 s, 27 H , 3 x *tert*-butyl) ppm. The signals were assigned using the data of **27a** as reference.

IR (film): $\tilde{\nu}$ = 3352 (ν NH), 2977 (ν_{as} CH_2), 2933 (ν_s CH_2), 1715, 1694 (ν C=O), 1613, 1590 (ν and δ ring), 1537 (ν_{as} NO_2), 1456, 1394 (δ $C(CH_3)_3$), 1456 (ν C–N), 1368 (δ $C(CH_3)_3$), 1304 (ν_s NO_2), 1159 (ν C–O) cm^{-1}.

E33: [α,5-bis-(*tert*-butoxycarbonyl)-2-nitrobenzyl] acetate (27a):



This substance was prepared as previously described for **26** (**E31**) (yield: 63%).

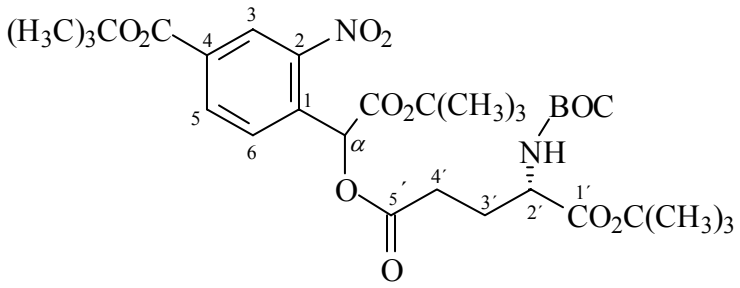
Mass (m/e): 322 [M-(OC(CH$_3$)$_3$)], 266, 238, 221, 219, 179, 149, 57 (B), 43

^{1}H-NMR (CDCl$_3$, 500 MHz) δ = 8.22 (d, $^4J_{HH}$ = 1.9 Hz, 1 H, Ar-6-H), 8.10 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.9, 1 H, Hz, Ar-4-H), 8.02 (d, $^3J_{HH}$ = 8.5 Hz, 1 H, Ar-6-H), 6.80 (s, 1 H, α-H), 2.22 (s, 3 H, COCH$_3$), 1.62, 1.42 (2s, 9 H each, C(CH$_3$)$_3$) ppm.

^{13}C-NMR (CDCl$_3$, 125 MHz) δ = 169.20 (1‘-C), 166.03 (Alk-COO-C(CH$_3$)$_3$), 163.36 (Aryl-COO-C(CH$_3$)$_3$), 150.58 (Ar-2-C), 136.35 (Ar-5-C), 130.57 (Ar-6-C), 130.29 (Ar-1-C), 128.56 (Ar-3-C), 124.95 (Ar-4-C), 83.97 (Ar—CO–O<u>C</u>(CH$_3$)$_3$), 82.82 (Alk–CO–O<u>C</u>(CH$_3$)$_3$), 70.25 (α-C), 28.14 (Ar–CO–OC(<u>C</u>H$_3$)$_3$), 27.82 (Alk–CO–OC(<u>C</u>H$_3$)$_3$), 20.55 (2´-C) ppm. The signals were assigned using COSY.

IR (film): $\tilde{\nu}$ = 2985, (ν_{as} CH alkyl), 2938 (ν_s CH alkyl), 1747, 1700 (ν C=O), 1608, 1591 (ν and δ ring), 1539 (ν_{as} NO$_2$), 1456, 1395 (δ C(CH$_3$)$_3$), 1306 (ν_s NO$_2$), 1161, (ν C–O) cm^{-1}.

E34: 4-*O*-[α,4-bis-(*tert*-butoxycarbonyl)-2-nitrobenzyl]-*N*-(*tert*-butoxycarbonyl)-1-*O*-*tert*-butyl-L-glutamic acid (28):



This substance was prepared as previously described for **26** (**E31**). The product was purified by column chromatography (silica gel, n-hexane/ethyl acetate 3:1) yielding 59% of a roughly 1:1.2 mixture of diastereomers (estimated from the ^{1}H-NMR integrals of the benzylic proton) as a pale brown viscous oil.

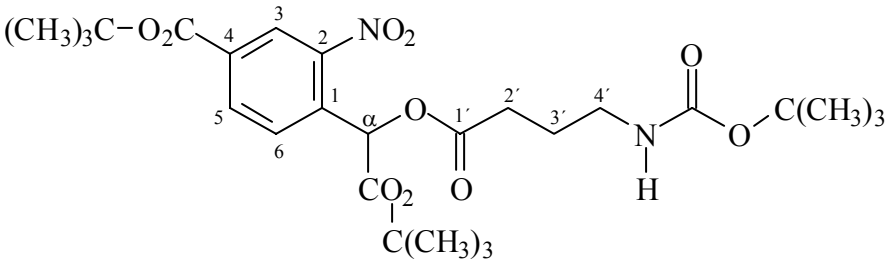
Anal. for $C_{31}H_{46}N_2O_{12}$ Calc.: C, 58.30; H, 7.20; N, 4.39; Found: C, 58.44; H, 7.18; N, 4.16.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.554 & 8.550 (2d, $^{4}J_{HH}$ = 2.0 & 1.9 Hz, 1 H, Ar-3-H), 8.24 & 8.22 (2 dd, $^{3}J_{HH}$ = 8.1 & $^{3}J_{HH}$ = 8.1, $^{4}J_{HH}$ = 2.0 & $^{4}J_{HH}$ = 1.8 Hz, 1 H, Ar-5-H), 7.74 & 7.71 (2 d, $^{3}J_{HH}$ = 8.1 $^{3}J_{HH}$ = 8.1, 1 H, Ar-6-H), 6.82 & 6.81 (2s, Integration 1:1.2, α-H), 5.16 & 5.1 (2 d, $^{3}J_{HH}$ = 7.9 Hz & 7.8 Hz, 1 H, N-H), 4.25 - 4.18 (m, 1 H, 2´-H), 2.68 - 2.45 (m, 2 H, 4´-H), 2.26 - 2.17, 2.02 - 1.89 (2 m, 2 H, 3´-H), 1.62 (1 s, 9 H, BOC), 1.47, 1.43, 1.39, 1.38 (4 s, 27 H, 3 x $COOC(CH_3)_3$) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 171.57 & 166.03 (5'-C, 1'-C), 163.46 (Alk-COO-*tert*-Butyl) 163.41 (Ar-COO-*tert*-Butyl), 155.73 (NH–CO–O), 148.52 (Ar-2-C), 134.09 (Ar-5-C), 131.41 (Ar-4-C), 129.56 (Ar-1-C), 129.51 (Ar-3-C), 126.17 (Ar-6-C), 84.40 & 84.32 (Ar-CO-O$\underline{C}(CH_3)_3$), 82.76 & 82.73 (β-C-O$\underline{C}(CH_3)_3$ and 1'-C-O$\underline{C}(CH_3)_3$), 80.23 (NH-CO-O$\underline{C}(CH_3)_3$), 70.56 (α-C), 53.77 & 53.77 (2'-C), 30.64 & 30.40 (4'-C), 28.69 (NH-CO-OC($\underline{C}H_3)_3$), 28.48 (Ar-CO-OC($\underline{C}H_3)_3$), 28.37 & 28.21 ((Alk-CO-OC($\underline{C}H_3)_3$ and 1'-CO-OC($\underline{C}H_3)_3$), 28.10 (3´-C) ppm.

IR (film): $\tilde{\nu}$ = 3433 (ν NH), 2979 (ν_{as} CH_2), 2935 (ν_s CH_2), 1714 (ν C=O), 1622 (ν ring), 1539 (ν_{as} NO_2), 1504, 1370 (δ C-H), 1300 (ν_s NO_2), 1153 (ν C–O) cm^{-1}.

E35: *O*-[α,4-bis-(*tert*-butoxycarbonyl)-2-nitrobenzyl]-*N*-(*tert*-butoxycarbonyl)-γ-amino butyric acid (29):



This substance was prepared as previously described for **26** (**E31**). The product was separated from the starting materials via column chromatography (silica gel, n-hexane/ethyl acetate 3:1) to yield 82% of **29** as yellow oil.

Anal. for $C_{26}H_{38}N_2O_{10}$ Calc.: C, 57.98; H, 7.11; N, 5.20; Found: C, 57.82; H, 7.26; N, 5.27.

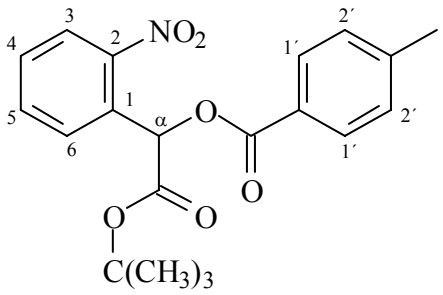
^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 8.54 (d, $^4J_{HH}$ = 1.9 Hz, 1 H, Ar-3-H), 8.22 (dd, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 1.9 Hz, 1 H, Ar-5-H), 7.71 (d, $^3J_{HH}$ = 8.2 Hz, 1 H, Ar-6-H), 6.78 (s, 1 H, α-H), 4.65 (s broad, 1 H, N-H), 3.20 (qua (broad), $^3J_{HH}$ = $^3J_{HH}$ = 6.4 Hz, 2 H, 4′-H), 2.55 & 2.49 (2dt, $^2J_{HH}$ = 16.2 $^3J_{HH}$ = 7.3 Hz, $^3J_{HH}$ = 7.1 Hz, 2 H, 2′-H), 1.88 (dt, $^3J_{HH}$ = $^3J_{HH}$ = 7.12 Hz, 2 H, 3′-H), 1.55, 1.37, 1.30 (3 s, 9 H each, 3 x $C(CH_3)_3$) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 171.93 (1′-C), 166.20 (Alk-COO-*tert*-Butyl), 163.44 (Ar-COO-*tert*-Butyl), 156.38 (NH-CO-O), 148.52 (Ar-2-C), 134.08 & 133.74 (Ar-5-C, Ar-4-C), 129.62 (Ar-1-C), 126.27 (Ar-3-C), 122.75 (Ar-6-C), 84.40 & 83.22 & 79.98 (3 x $\underline{C}OOC(CH_3)_3$), 70.45 (α-C), 60.79 (4′-C), 40.07 (2′-C), 31.97 (3′-C), 28.78 & 28.48 & 28.21 (3 x $C(\underline{C}H_3)_3$) ppm.

IR (film) $\tilde{\nu}$ = 3375 (ν N-H), 2980 (ν_{as} C-H), 2935 (ν_s C-H), 1721 (ν C=O), 1622, 1477 (ν and δ ring), 1537 (δ NH), 1456 (ν C-N), 1395, 1370 (δ $C(CH_3)_3$), 1301, 1152 (ν C–O), 1256 (ν C–N) cm^{-1}.

E36: *tert*-Butyl α-(4-methylphenylcarbonyloxy)-α-(2-nitrophenyl)acetate

or α-(*tert*-Butoxycarbonyl)-2-nitrobenzyl toluate (30)



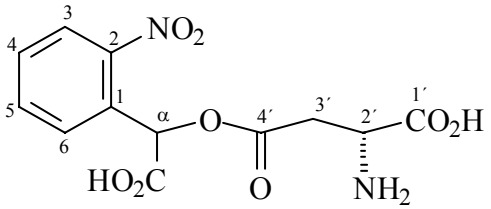
This substance was prepared as previously described for **25** (**E30**). The product mixture was then filtered through a silica gel column (n-hexane/ethyl acetate 4:1) to yield (99%) **30** as yellow oil.

Anal. for $C_{20}H_{21}NO_6$ Calc.: C, 64.68; H, 5.70; N, 3.77; Found: C, 64.40; H, 5.73; N, 3.60.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.05 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.3 Hz, 1 H, Ar-3-H), 8.00 (dd, $^3J_{HH}$ = 8.3, $^4J_{HH}$ = 1.9 Hz, 2 H, Ar-1´-H), 7.75 (dd, $^3J_{HH}$ = 7.9, $^4J_{HH}$ = 1.2 Hz, 1 H, Ar-6-H), 7.66 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.3 Hz, 1 H, Ar-5-H), 7.54 (ddd, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.5 Hz, 1 H, Ar-4-H), 7.27 (m, > 2 H, Ar-2´-H and $CHCl_3$), 6.97 (s, 1 H, α-H), 2.42 (s, 3 H, Ar-CH_3), 1.42 (s, 9 H, $C(CH_3)_3$) ppm.

IR (film): $\tilde{\nu}$ = 2982 (ν_{as} CH alkyl), 2934 (ν_s CH alkyl),1749, 1722 (ν C=O), 1612, 1579 (ν and δ ring), 1534 (ν_{as} NO_2), 1456 (ν_s CH), 1410, 1394 (δ $C(CH_3)_3$), 1355 (ν_s NO_2), 1151 (ν C–O) cm^{-1}.

E37: 4-*O*-(α-carboxy-2-nitrobenzyl)-*R*-aspartic acid (31):



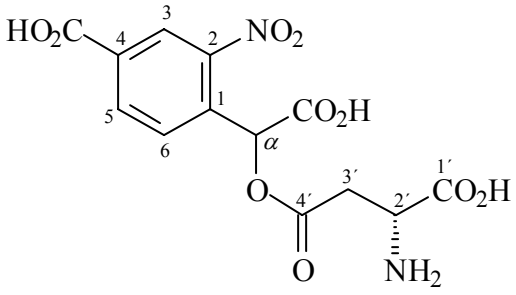
A solution of 0.24 g (0.45 mmol) ester **25** in a mixture of 11 ml dichloromethane and 4 ml trifluoroacetic acid (TFA) was stirred at rt under nitrogen for 16 h. The mixture was concentrated under reduced pressure, 20 ml toluene was added and the solvent was evaporated again. This procedure was repeated once and the residual brown oil was purified on sephadex LH 20 with water. After liophylization 0.13 g (0.30 mmol, 65%) caged aspartic acid **31** was obtained as 1:1 mixture of diastereomers (estimated from the ^{1}H-NMR integrals of the benzylic proton) as white solid, mp 112 - 114 ºC.

Anal. for $C_{16}H_{20}N_2O_8$ & 1/3 CF_3COOH Calc.: C, 49.26; H, 5.04; N, 6.89 Found: C, 49.43; H, 4.96; N, 7.08.

^{1}H-NMR (D_2O, 300 MHz) δ = 8.2 – 8.0 (m, 1 H, Ar-3-H), 7.9 – 7.7 (m, 1 H, Ar-5-H), 7.7 – 7.6 (m, 2 H, Ar-4-H, Ar-6-H), 6.69, 6.67 (2 s, 1 H, Ar-CH), 4.6 – 4.5 (m, 1 H, NH-C<u>H</u>), 3.3 – 3.1 (m, 2 H, CH_2) ppm.

IR (KBr): $\tilde{\nu}$ = 3500-2400 (ν OH & NH & Ar-H & CH), 1708 (ν C=O), 1600, 1578 (ν ring), 1522 (ν_{as} NO_2), 1480 (ν_s CH_2, CH), 1351 (ν_s NO_2), 1239 (ν C–O) cm^{-1}.

E38: 4-*O*-(α,4-Biscarboxy-2-nitrobenzyl)-*R*-aspartic acid (32):

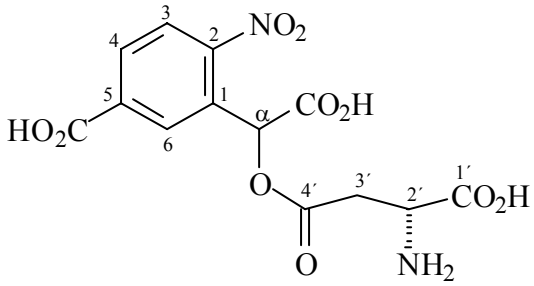


This substance was prepared as previously described for **31** in **E37** (yield: 88%) as a roughly 1.1:1 mixture of diastereomers (estimated from the ^{1}H-NMR integrals of the benzylic proton) as a white and very hygroscopic powder, mp 128 - 130 °C.

Anal. for $C_{13}H_{12}N_2O_{10}$, $2H_2O$ Calc.: C, 39.80; H, 4.11; N, 7.14; Found: C, 39.60; H, 4.08; N, 6.97 .

^{1}H-NMR (D_2O, 300 MHz) δ = 8.48 & 8.47 (2 d, $^{4}J_{HH}$ = 1.8 Hz and 1.7 Hz, 1 H, Ar-3-H), 8.19 & 8.18 (2 dd, $^{3}J_{HH}$ = 8.1 Hz, $^{3}J_{HH}$ = 8.1 Hz, $^{4}J_{HH}$ = 2.0 Hz, $^{4}J_{HH}$ = 2.0 Hz, 1 H, Ar-5-H), 7.73 and 7.73 (2 d, $^{3}J_{HH}$ = 8.2 Hz, $^{3}J_{HH}$ = 8.2 Hz, 1 H, Ar-6-H), 6.73 & 6.70 (2s, (Integration 1.1:1), 1 H, α-H), 4.36 & 4.35 (2 dd, $^{3}J_{HH}$ = 6.3 Hz, $^{3}J_{HH}$ = 5.0 Hz and $^{3}J_{HH}$ = 6.3 Hz, $^{3}J_{HH}$ = 5.0 Hz 1 H, 2'-H), 3.300 & 3.296 & 3.22 & 3.21 (4 dd, $^{2}J_{HH}$ = 18.0 Hz, $^{3}J_{HH}$ = 6.4 Hz & $^{2}J_{HH}$ = 18.0 Hz, $^{3}J_{HH}$ = 5.1 Hz & $^{2}J_{HH}$ = 18.1 Hz, $^{3}J_{HH}$ = 6.4 Hz & $^{2}J_{HH}$ = 18.1 Hz, $^{3}J_{HH}$ = 5.1 Hz, 2 H, 3'-H) ppm.

E39: 4-*O*-(*a*,5-Biscarboxy-2-nitrobenzyl)-*R*-aspartic acid (**33**):



This substance was prepared as previously described for **31** in **E37** (yield 82%) as a roughly 1.4:1 mixture of diastereomers (estimated from the ^{1}H-NMR integrals of the benzylic proton, see NMR spectrum on the next page) as a white and very hygroscopic powder, mp 136-138 °C.

Anal. for $C_{13}H_{12}N_2O_{10}$, H_2O Calc.: C, 41.72; H, 3.77; N, 7.48; Found: C, 41.74; H, 3.87; N, 7.57 .

^{1}H-NMR (D_2O, 500 MHz) δ = 8.14 (s (broad), 1 H, Ar-6-H), 8.12 (dd (broad), $^{3}J_{HH}$ = 8.6 Hz, $^{4}J_{HH}$ = 1.5 Hz, 1 H, Ar-4-H), 8.09 (d (broad), $^{3}J_{HH}$ = 8.6 Hz, 1 H, Ar-3-H), 6.61 & 6.59 (2 s, (Integration 1:1.4), 1 H, α-H), 4.12 (2 dd, $^{3}J_{HH}$ = 6.0 Hz, $^{3}J_{HH}$ = 6.0 Hz, 1 H, 2'-H), 3.21- 3.08 (m, 2 H, 3'-H) ppm.

^{13}C-NMR (D_2O, 125 MHz): δ = 172.08 (Alk-COOH), 170.53 (Ar-COOH), 168.53 & 167.67 (1´-C, 4´-C), 150.44 (Ar-2-C), 136.22 (Ar-5-C), 131.93 (Ar-6-C), 130.72 (Ar-1-C), 130.01 (Ar-3-C), 126.06 (Ar-4-C), 72.61 (α-C), 52.64 (2´-C), 25.18 (3´-C) ppm.

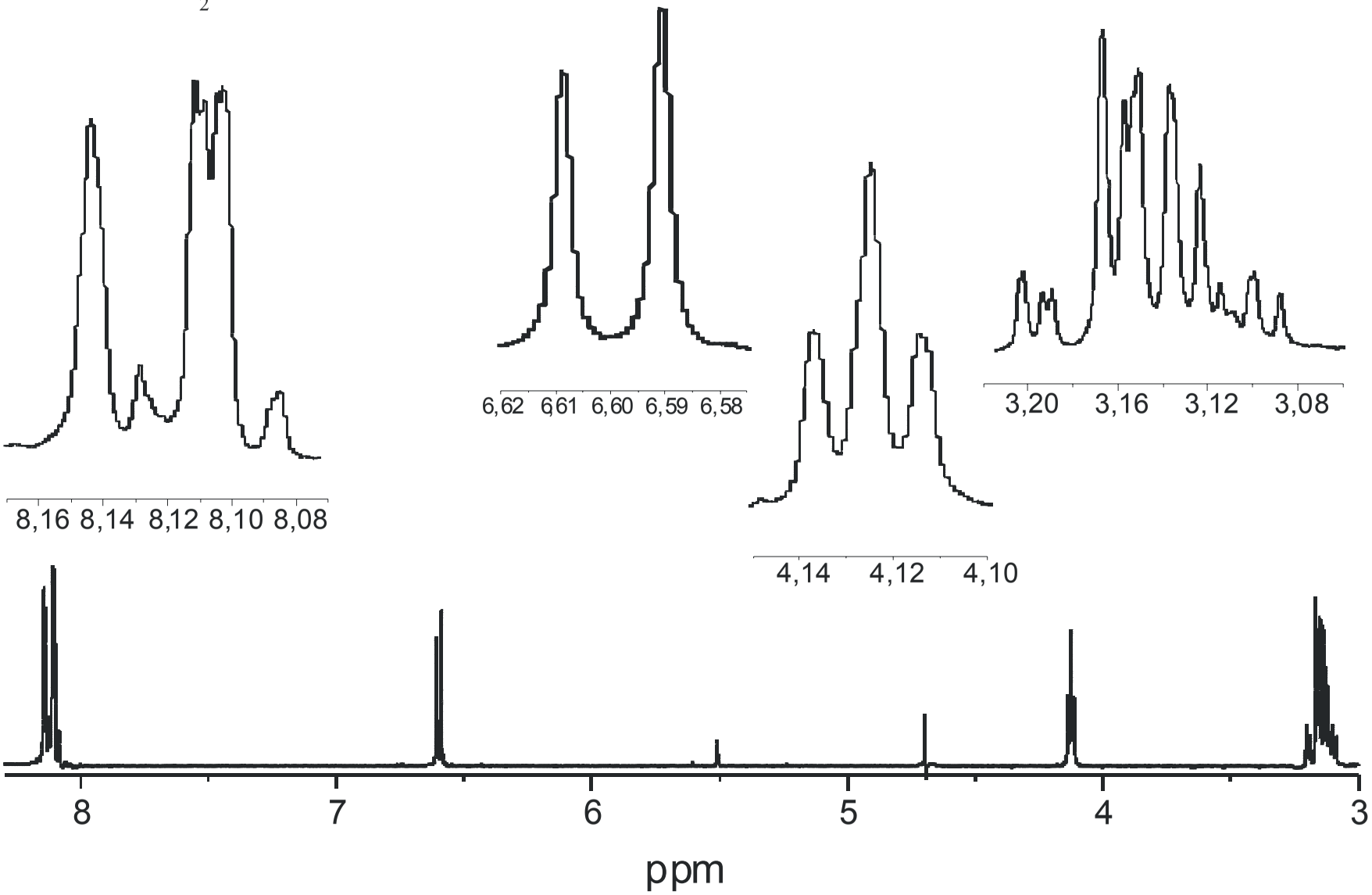
IR (KBr): $\tilde{\nu}$ = 3600-2500 (ν OH & NH & Ar-H & CH), 1730, 1671 (ν C=O), 1534 (ν_{as} NO_2), 1458 (ν_s CH_2, CH), 1367 (ν_s NO_2), 1190 (ν C–O) cm^{-1}.

α,5-DCNB -D- Asp (**33**)

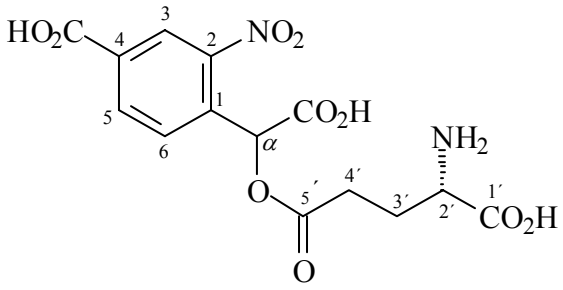
500MHz in D_2O

α-H

Integral ~ 1 : 1.4



E40: 4-*O*-(α,4-Biscarboxy-2-nitrobenzyl)-L-glutamic acid (34):



A solution of 400 mg (0.630 mmol) **28** in a mixture of 11 ml dichloromethane and 4 ml trifluoroacetic acid (TFA) was stirred at rt under nitrogen for 18 h. The mixture was concentrated under reduced pressure, 10 ml toluene was added and the solvent was evaporated again. The procedure was repeated once and the residual solid was purified on sephadex LH 20 with water as eluant giving 173 mg (0.460 mmol, 73%) as a roughly 1:1 mixture of diastereomers of **34** (estimated from the ^{1}H-NMR integrals of the benzylic proton) as a white and very hygroscopic powder solid, mp 135 - 137 °C.

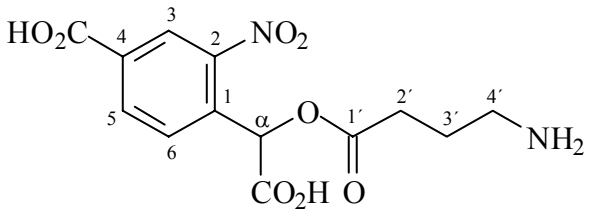
Anal. for $C_{14}H_{14}N_2O_{10}$, ½ CF_3CO_2H Calc. C, 42.17; H, 3.42; N, 6.56; Found: C, 42.35; H, 3.62; N, 6.57.

^{1}H-NMR (D_2O, 500 MHz): δ = 8.41 & 8.40 (2d, $^{4}J_{HH}$ = $^{4}J_{HH}$ =1.6 Hz, 1H, Ar-3-H), 8.13 & 8.12 (2 dd, $^{3}J_{HH}$ = $^{3}J_{HH}$ = 8.1 Hz, $^{4}J_{HH}$ = $^{4}J_{HH}$ = 1.7 Hz, 1H, Ar-5-H), 7.68 & 7.67 (2 d, $^{3}J_{HH}$ = $^{3}J_{HH}$ =8.1 Hz, 1H, Ar-6-H), 6.63 & 6.62 (2s, (Integration 1:1), 1H, α-H), 4.01 & 4.00 (2 dd, $^{3}J_{HH}$ = 6.7 Hz, $^{3}J_{HH}$ = 6.6 Hz and $^{3}J_{HH}$ = 6.8 Hz, $^{3}J_{HH}$ = 6.78 Hz, 1H, 2′-H), 2.80-2.62 (m, 2H, 3′-H), 2.75-2.11 (m, 2H, 4′-H) ppm.

^{13}C-NMR (D_2O, 125 MHz) δ = 173.04 (Alk-COOH), 172.99 (Ar-COOH), 172.14 & 172.12 (1′-C), 171.94 (5′-C), 134.87 (Ar-2-C), 134.36 & 134.30 (Ar-5-C), 132.38 (Ar-4-C), 131.08 (Ar-1-C), 129.70 (Ar-3-C), 126.54 (Ar-6-C), 72.08 (α-C), 52.60 & 52.46 (2′-C), 29.87 & 29.75 (4′-C), 25.18 (3′-C) ppm.

IR (KBr): $\tilde{\nu}$ = 3600-2500 (ν OH & NH & Ar-H & CH), 1731 (ν C=O), 1622 (ν ring), 1539 (ν_{as} NO_2), 1505 (ν_s CH_2, CH), 1356 (ν_s NO_2), 1191 (ν C–O) cm^{-1}.

E41: 4-*O*-(α,4-Biscarboxy-2-nitrobenzyl)-γ-aminobutyric acid (35):

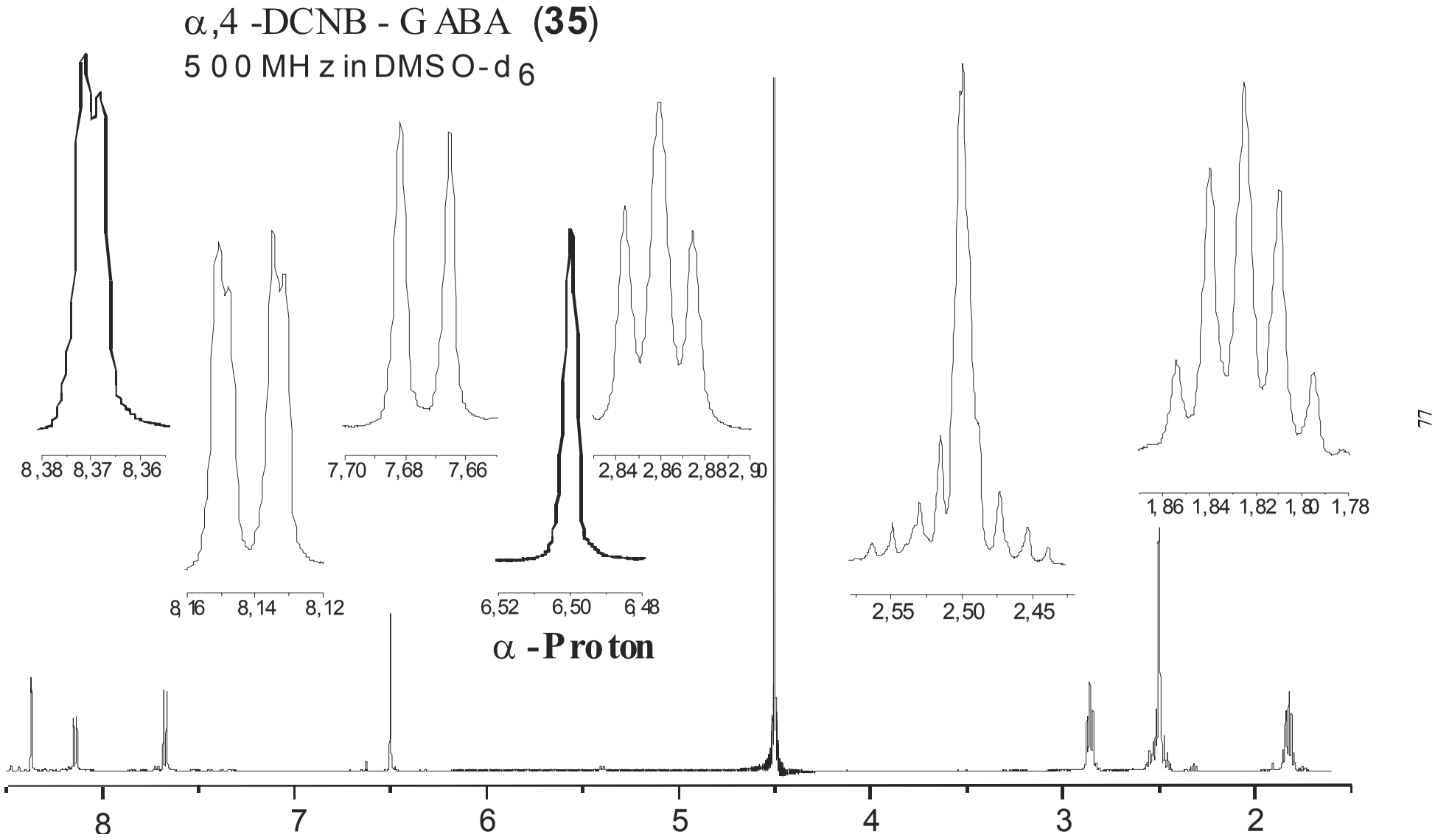


This substance was prepared as previously described for **31** in **E37** (yield 88%) as a white and very hygroscopic powder, mp 112 - 114 °C.

Anal. for $C_{13}H_{14}N_2O_8$, H_2O & ½ CF_3COOH Calc.: C, 41.90; H, 4.14; N, 6.98; Found: C, 42.11; H, 4.13; N, 6.84.

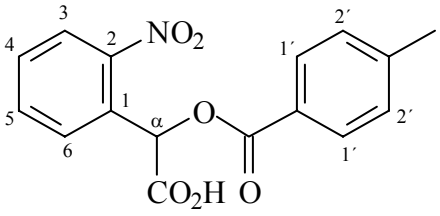
^{1}H-NMR (DMSO-d_6, 500 MHz) δ = 8.37 (d, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-3-H), 8.14 (dd, $^3J_{HH}$ = 8.2, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-5-H), 7.67 (d, $^3J_{HH}$ = 8.2 Hz, 1 H, Ar-6-H), 6.50 (s, 1 H, α-H), 4.6 (s broad, H_2O & NH_2), 2.86 (t, $^3J_{HH}$ = 7.5 Hz, 2 H, 4´-H), 2.60 – 2.40 (m, > 2 H, 3´-H & DMSO), 1.83 (dt, $^3J_{HH}$ = 7.5 Hz, 2 H, 2´-H) ppm (NMR spectrum is shown on the next page).

IR (KBr): $\tilde{\nu}$ = 3600-2500 (ν OH & NH & Ar-H & CH), 1725 (ν C=O), 1645 (ν ring), 1538 (ν_{as} NO_2), 1359 (ν_s NO_2), 1189 (ν C–O) cm^{-1}.


α,4 -DCNB - GABA (35)
500 MHz in DMSO-d6
8,38 8,37 8,36
8,16 8,14 8,12
7,70 7,68 7,66
6,52 6,50 6,48
2,84 2,86 2,88 2,90
2,55 2,50 2,45
1,86 1,84 1,82 1,80 1,78
α -Proton
8
7
6
5
4
3
2

E42: α-(4-Methylphenylcarboxy-2-nitrophenyl)acetic acid

or α–Carboxy-2-nitrobenzyl toluate (36a)



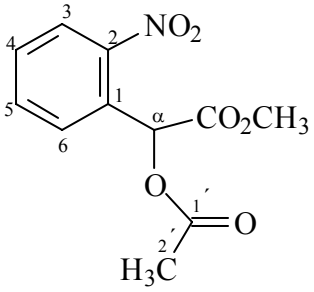
This substance was prepared as previously described for **31** (**E37**). The mixture (soluble in chloroform) was filtered through a silica gel column (acetone/methanol 5:1) and then added dropwise to diethyl ether to yield (86%) **36** as white precipitate, mp 148 - 150 °C.

Anal. for $C_{16}H_{13}NO_6$ Calc.: C, 60.95; H, 4.16; N, 4.44; Found: C, 60.83; H, 3.98; N, 4.40.

^{1}H-NMR (DMSO-d_6, 200 MHz) δ = 8.17-7.45 (m, 6 H, Ar-1, 1´, 3, 4, 5, 6-H), 7.36 (d, $^3J_{HH}$=8.02, 2 H, Ar-2´-H), 5.65 (s, 1 H, α-H), 3.35 (b, water and acidic-H), 2.39 (s, 3 H, Ar-CH_3) ppm.

IR (KBr): $\tilde{\nu}$ = 3020 (ν C-H aryl), about 3000 (ν OH), 2982 (ν_{as} CH alkyl), 1714 (ν C=O), 1613 (ν and δ ring), 1530 (ν_{as} NO_2), 1415 (ν_s CH), 1415 (δ $C(CH_3)_3$), 1352 (ν_s NO_2), 1170 (ν C–O) cm^{-1}.

E43: [α-(Methoxycarbonyl)-2-nitrobenzyl] acetate (37):



A mixture of 0.10 ml (0.10 g, 1.66 mmol) acetic acid , 0.30 g (1.09 mmol) methyl α-bromo-α-(2-nitrophenyl)acetate (**21a**) and 0.26 g (1.71 mmol) 1,8-diazabicyclo[5.4.0]undec-7-ene

(DBU) in 20 ml dry benzene was heated under reflux for 1 h. The reaction was followed with TLC (n-hexane/diethyl ether 2:1). The mixture was allowed to cool to rt and 20 ml water and 20 ml ethyl acetate were added, the organic layer was removed and the aqueous layer was washed with ethyl acetate (20 ml). The combined organic layers were concentrated under reduced pressure to yield a pale brown oil. This oil was dissolved in 3 ml n-hexane/diethyl ether (2:1) and filtered through a silica gel column, dried over sodium sulfate and concentrated to give 0.17 g (0.69 mmol, 63%) of compound **37** as yellow oil.

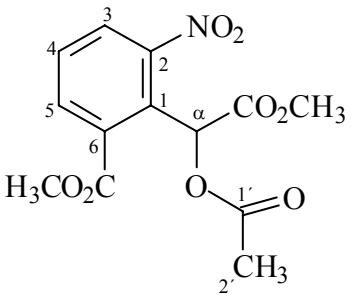
Anal. for $C_{11}H_{11}NO_6$ Calc.: C, 52.18; H, 4.38; N, 5.53; Found: C, 52.14; H, 4.27; N, 5.55.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.04 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.3 Hz, 1 H, Ar-3-H), 7.67 (ddd, $^3J_{HH}$ = 7.7 Hz, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.3 Hz, 1 H, Ar-5-H), 7.63 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H, Ar-6-H), 7.55 (ddd, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.8 Hz, 1 H, Ar-4-H), 6.84 (s, 1 H, α-H), 3.75 (s, 3 H, OCH_3), 2.20 (s, 3 H, 2´-H) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 168.34 (Alk-CO–OCH_3), 167.07 (1´-C), 147.11 (Ar-2-C), 132.32 (Ar-1-C), 128.96, 128.60, 128.38 (Ar-5-C & Ar-3-C & Ar-4-C), 124.29 (Ar-6-C), 68.83 (α-C) 52.77 (CO–OCH_3), 19.56 (2´-C) ppm.

IR (film): $\tilde{\nu}$ = 2960 (ν_{as} CH alkyl), 1748 (ν C=O), 1612, 1580 (ν and δ ring), 1531 (ν_{as} NO_2), 1437 (δ CH), 1219 (ν_s NO_2), 1061 (ν C–O) cm^{-1}.

E44: [α,6-(dimethoxycarbonyl)-2-nitrobenzyl] acetate (38):



This substance was prepared as previously described for **26** (**E31**). The product was purified by column chromatography (silica gel, n-hexane/diethyl ether 1:3).

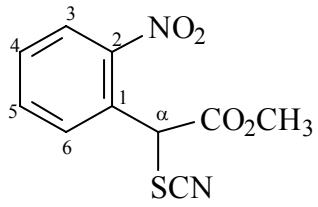
Anal. for $C_{12}H_{13}NO_8$ Calc.: C, 50.17; H, 4.21; N, 4.50; Found: C, 50.02; H, 4.07; N, 4.40.

^{1}H-NMR ($CDCl_3$, 200 MHz) δ = 8.10 (dd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-3-H), 7.98 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ =1.4 Hz, 1 H, Ar-5-H), 7.60 (dd, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 8.0 Hz, 1 H, Ar-4-H), 5.30 (s, 1 H, α-H), 3.95 (s, 3 H, Ar–CO–OCH_3), 3.81 (s, 3 H, Alk-CO–OCH_3), 2.09 (s, 3 H, 2´-H) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 173.04 (Alk-CO–OCH_3), 169.67 (Ar-COOH), 168.75 (1´-C), 148.37 (Ar-2-C), 134.32 (Ar-6-C), 130.66 (Ar-1-C), 129.74, 129.30, 125.34 (Ar-5-C & Ar-3-C & Ar-4-C), 69.70 (α-C), 60.11, 58.55 (CO–OCH_3), 21.10 (2´-C) ppm.

IR (film): $\tilde{\nu}$ = 3090 (ν C-H aryl), 2957 (ν_{as} CH alkyl), 1748 (ν C=O), 1607, 1577 (ν and δ ring), 1539 (ν_{as} NO_2), 1436 (δ CH), 1218 (ν_s NO_2), 1061 (ν C–O) cm^{-1}.

E45: Methyl [α-(2-nitrophenyl)-α-thiocyanato]acetate (39):



A mixture of 0.21 g (2.2 mmol) potassium thiocyanate and 0.40 g (1.5 mmol) methyl α-bromo-α-(2-nitrophenyl)acetate (**21a**) in 15 ml acetone was stirred for 10 min at rt (followed by TLC, n-hexane/diethyl ether 2:1). The mixture was concentrated and treated with 25 ml n-hexane/diethyl ether (1:1) mixture. The solution was dried over sodium sulfate and the solution was concentrated to yield 0.32 g (1.3 mmol, 87%) **39** as yellow oil.

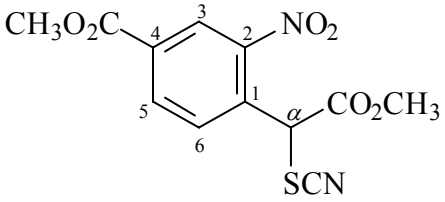
Anal. for $C_{10}H_8N_2O_4S$ Calc.: C, 47.62; H, 3.20; N, 11.11; Found: C, 47.49; H, 3.14; N, 11.02.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.25 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.4 Hz, 1 H, Ar-3-H), 7.77 (td, $^3J_{HH}$ = 7.6 Hz, $^4J_{HH}$ =1.4 Hz, 1 H, Ar-5-H), 7.71 (ddd, $^3J_{HH}$ = 8.2 Hz, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.5 Hz, 1 H, Ar-4-H), 7.60 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 1.5 Hz, 1 H, Ar-6-H), 5.67 (s, 1 H, α-H) 3.82 (s, 3 H, Ar–CO–OCH_3) ppm.

^{13}C-NMR ($CDCl_3$, 125 MHz) δ = 166.32 Alk–<u>C</u>O–OCH_3), 146.62 (Ar-2-C), 134.73 (Ar-1-C), 132.47 (Ar-3-C), 131.04 (Ar-5-C), 129.66 (Ar-4-C), 126.42 (Ar-6-C), 110.90 (-S-C≡N), 65.84 (α-C) 52.77 (CO–O<u>C</u>H_3) ppm.

IR (film): $\tilde{\nu}$ = 2957 (ν_{as} CH alkyl), 2157 (ν -S=C=N), 1744 (ν C=O), 1609, 1578 (ν and δ ring), 1531 (ν_{as} NO_2), 1437 (δ CH), 1268 (ν_s NO_2), 1226 (ν C–O) cm^{-1}.

E46: Methyl [α-(4-methoxycarbonyl-2-nitrophenyl)-α-thiocyanato]acetate (40):



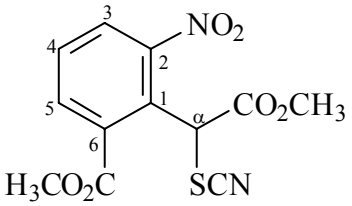
A mixture of 0.527 g (5.42 mmol) potassium thiocyanate and 1.20 g (3.61 mmol) methyl α-bromo-α-(2-nitrophenyl)acetate (**21b**) in 20 ml acetone was stirred at rt (followed by TLC, n-hexane/diethyl ether 3:1). After 4 h the mixture was concentrated at rt and filtered with a n-hexane/diethyl ether (3:1) mixture through a silica gel column. The remaining product was washed with diethyl ether from the column. The organic layer was dried over sodium sulfate and the solution was concentrated to yield 0.92 g (3.0 mmol, 82%) **40** as yellow oil.

Anal. for $C_{12}H_{10}N_2O_6S$ Calc.: C, 46.45; H, 3.25; N, 9.03; Found: C, 46.56; H, 2.99; N, 8.89.

^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 8.85 (d, $^4J_{HH}$ = 1.8 Hz, 1 H, Ar-3-H), 8.38 (dd, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ =1.8, 1 H, Ar-5-H), 7.70 (d, $^3J_{HH}$ = 8.0 Hz, 1 H, Ar-6-H), 5.70 (s, 1 H, α-H), 4.01 (s, 3 H, Ar–CO–OCH_3), 3.83 (s, 3 H, Alk-CO–OCH_3) ppm.

IR (film): $\tilde{\nu}$ = 3093, 3008 (ν C-H aryl), 2957 (ν CH & CH_3), 2158 (ν -S=C=N), 1734 (ν C=O), 1622 (ν ring), 1538 (ν_{as} NO_2), 1437 (δ CH & CH_3), 1347 (ν_s NO_2), 1288, 1123 (ν C–O) cm^{-1}.

E47: Methyl [α-(6-methoxycarbonyl-2-nitrophenyl)-α-thiocyanato]acetate (41):



This substance was prepared as previously described for **40** (**E46**). After 24 h the product was purified by column chromatography (n-hexane/diethyl ether 1:3) to yield (68%) a yellow oil.

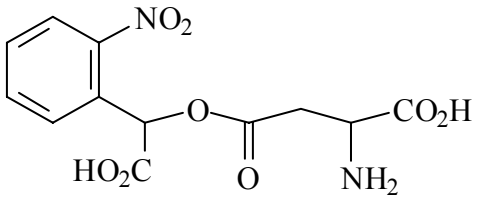
Anal. for $C_{12}H_{10}N_2O_6S$ Calc.: C, 46.45; H, 3.25; N, 9.03 Found: C, 45.60; H, 3.15; N, 8.80.

^{1}H-NMR ($CDCl_3$, 200 MHz) δ = 8.36 (dd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.5 Hz, 1 H, Ar-3-H), 8.27 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ =1.5 Hz, 1 H, Ar-5-H), 7.70 (dd, $^3J_{HH}$ = 8.3 Hz, $^3J_{HH}$ = 7.9 Hz, 1 H, Ar-4-H), 6.86 (s, 1 H, α-H), 4.0 (s, 3 H, Ar–CO–OCH_3), 3.81 (s, 3 H, Alk-CO–OCH_3) ppm.

IR (film): $\tilde{\nu}$ = 2957 (ν CH & CH_3), 2156 (ν -S=C=N), 1737 (ν C=O), 1620 (ν ring), 1536 (ν_{as} NO_2), 1436 (δ CH & CH_3), 1353 (ν_s NO_2), 1287, 1128 (ν C–O) cm^{-1}.

Appendix I

α-CNB-Caged aspartate



31

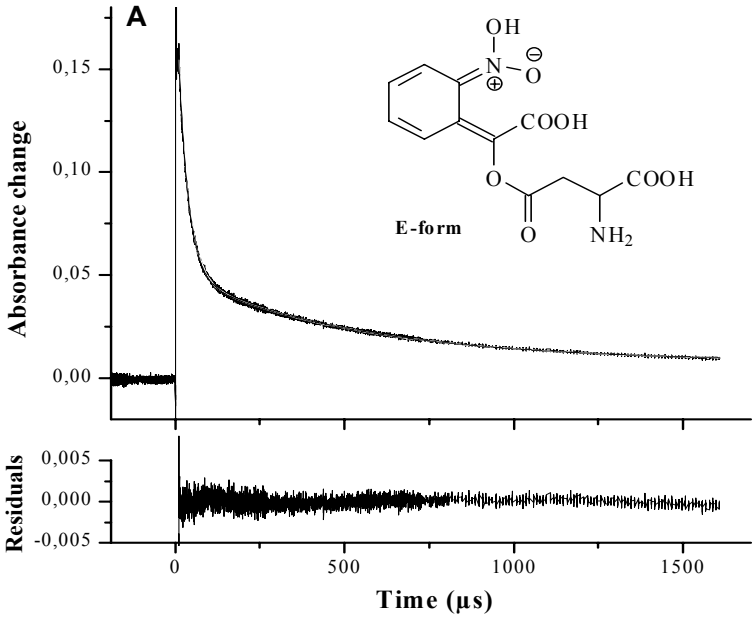


Figure 1: Transient absorbance change produced by 1 mM **31** after photolysis with a 10 ns, 308 nm laser flash. The wavelength of the analysis light was 430 nm with a path length of 10 mm (T = 22°C and pH 7.0 (100 mM phosphate buffer). The solid line represents the best fit to double exponential decay function. The lower panel shows the fitting residuals.

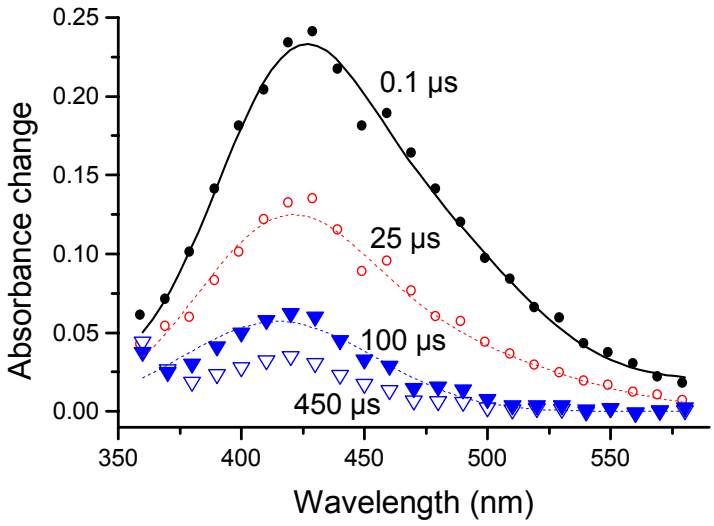


Figure 2: UV-visible spectrum of the transient absorbance change induced by a 308 nm laser puls of a mM **31** as a function of time (T = 22 °C and pH 7.0; 100 mM phosphate buffer). The spectra were recorded at times of 0.1, 25, 100 and 450 µs after laser flash.

pH k / 10^3 (s^{-1})	5	6	7	8	9	10
k_1 fast	62.8	48.9	40.1	31.6	21.8	16.9
k_2 slow	1.84	2.51	2.70	2.42	2.49	1.72

Table 1: Rate constants of the decay of the fast and slow exponential decay of the transient absorbance at different pH values (average of 3 experiments) are shown.

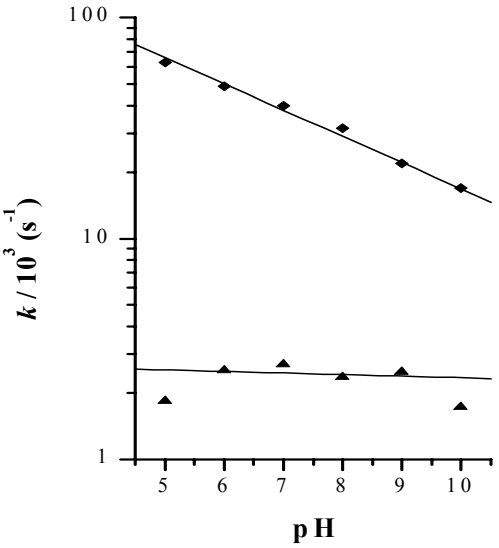


Figure 3: Rate constants of the decay of the fast (♦) and slow (▲) exponential decay of the transient absorbance as a function of pH (average of 3 experiments) are shown. The buffer solutions were acetate (pH 5.0), phosphate (pH 6.0, 7.0 and 8) and borate (pH 9.0 and 10.0) at concentrations of 100 mM, respectively (T = 22 °C).

pH	5	6	7	8	9	10
Φ	0.08	0.17	0.18	0.134	0.127	0.06

Table 2: Quantum yields for the release of D-aspartate from **31** at different pH values are shown.

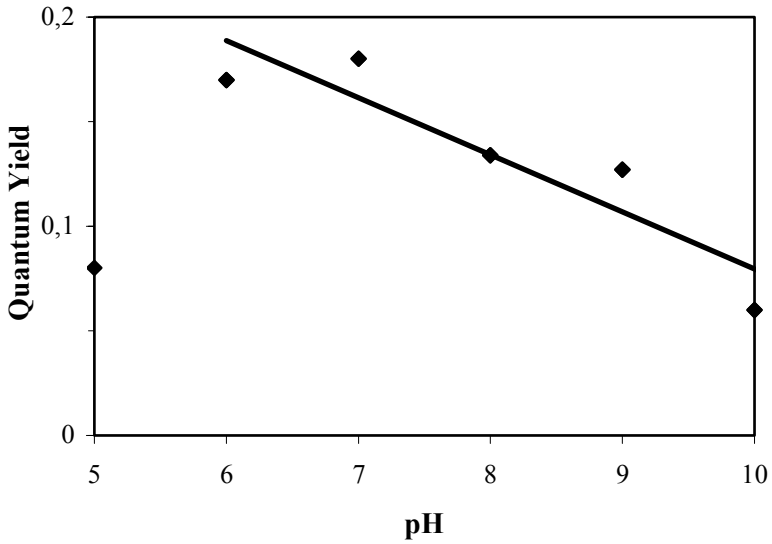
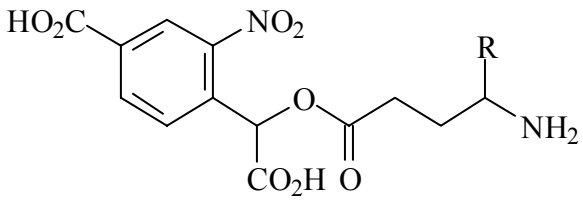


Figure 4: Dependence of the photolysis quantum yield on the pH of the solution (T = 22 °C). The buffer solutions were the same as in figures 2 and 3.

Appendix II

α,4-DCNB-Caged Glutamate (34) and GABA (35)



34 : R=CO_2H

35 : R=H

The photopysical properties of the caged L-glutamate **34** and the caged GABA **35** have been investigated by means of laser spectroscopy. The results, including those for the respective D-aspartate **32** are shown in table 1:

	t_1/μs	t_2/μs	Φ
32	110 (68%)	508 (32%)	0.14
34	77 (95%)	426 (5%)	0.10
35	57 (54%)	211 (46%)	0.16

Table 1. Overview over the time constants and quantum yields for caged compounds utilizing the α,4-DCNB protecting group. The observed rate constants and quantum yields for all three compounds are within the same range as one would expect for three systems which have the same caging group attached to the same functional group.

pH-Dependence of the rate constants of the fast (k_1) and the slow (k_2) exponential component of the decay of the *aci*-nitro intermediates **42** of *α*,4-DCNB caged glutamate **34** and *α*,4-DCNB caged GABA **35** are shown in figure 1 and 2. The determined rate (k) and time (t) constants are summarized in tables 2 and 3.

pH	k_1 [ms^{-1}] ♦	t_1/µs	k_2 [ms^{-1}] ▲	t_2/µs
4	13.64	73	4.63	216
5	25.19	40	5.28	189
6	19.91	50	3.52	284
7	13.07	77	2.35	426
8	12.06	83	2.04	490
9	11.69	86	1.77	565
10	10.66	94	1.53	654

Table 2: rate and time constants of the *aci*-nitro intermediate **42** of the **34**.

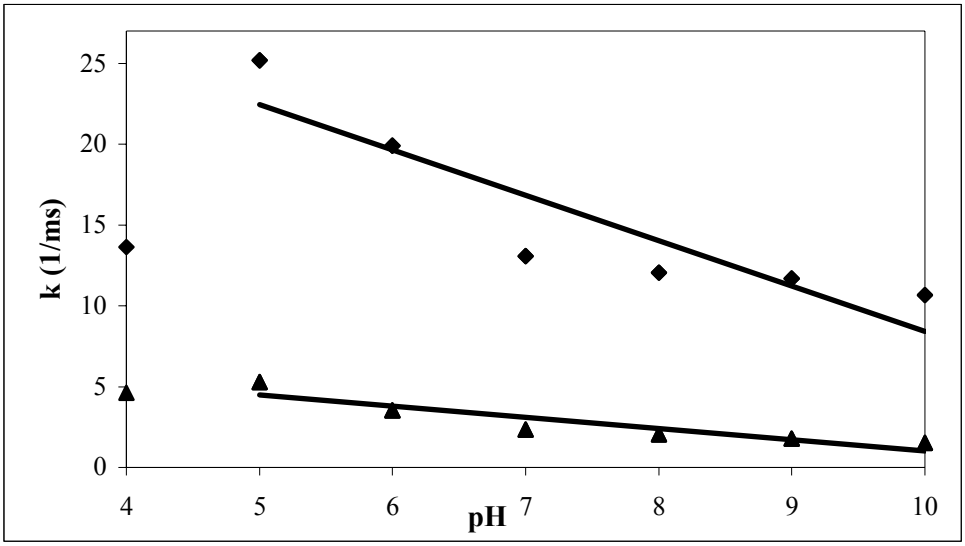


Figure 1: *α*,4-DCNB-L-Glutamate **(34):** A decrease in the rate constant below pH = 5 has been reported for caged compounds with an α-carboxy group[18, 19]. We have found this decrease for **34** already at pH = 5.

pH	k_1 [ms^{-1}] ♦	t_1/µs	k_2 [ms^{-1}] ▲	t_2/µs
4	63.61	16	10.22	98
5	40.30	25	6.55	153
6	35.22	28	6.65	150
7	17.54	57	4.74	211
8	15.92	63	4.25	235
9	13.79	11	3.94	254
10	9.97	10	2.80	357

Table 3: rate and time constants of the *aci*-nitro intermediate **42** of the **35**.

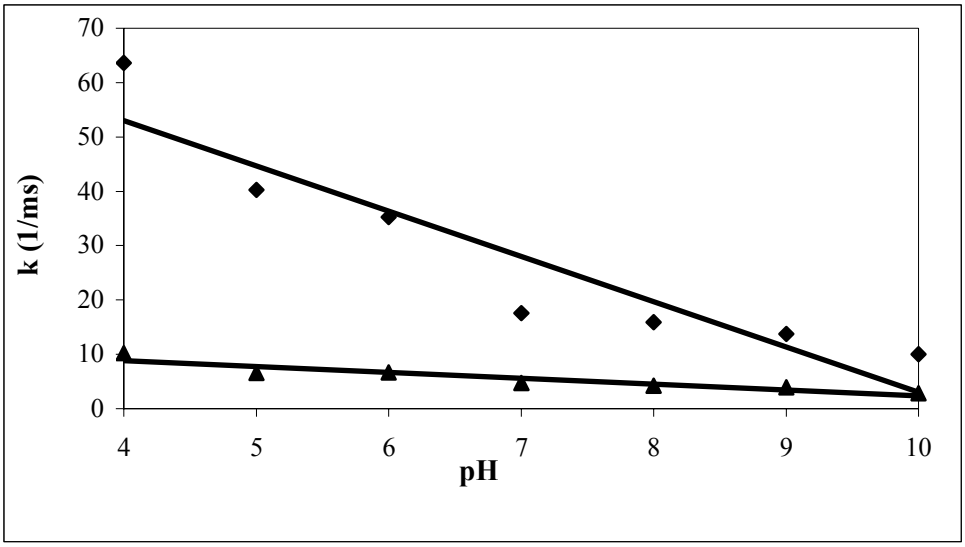
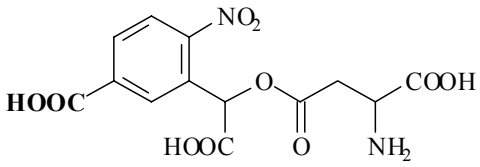


Figure 2: pH-Dependence of the rate constants of the fast (k_1) and the slow (k_2) of the decay of the *aci*-nitro intermediates **42** of α,4-DCNB caged GABA **35** are shown.

Appendix III

α,5-DCNB-Caged Aspartate (33)



33

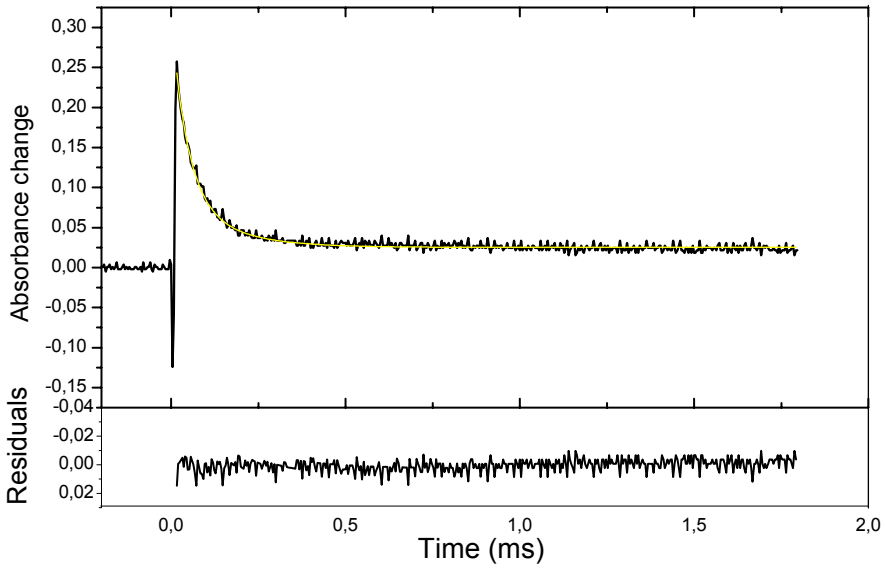


Figure 1: Transient absorbance change produced by 1 mM **33** after photolysis with a 10 ns, 308 nm laser flash. The wavelength of the analysis light was 430 nm with a path length of 10 mm (T = 22 °C and pH 7.0 (100 mM phosphate buffer). The solid line represents the best fit to the data whit a double exponential decay function. The lower panel shows the fitting residuals.

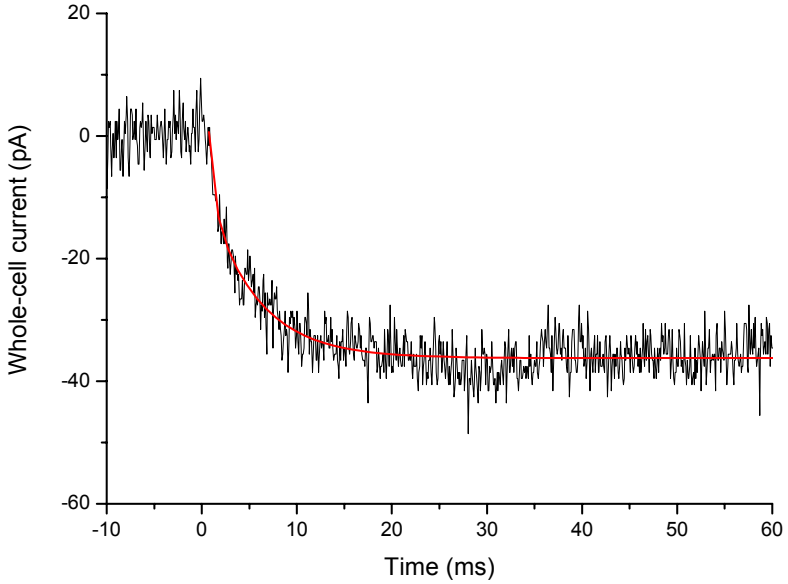
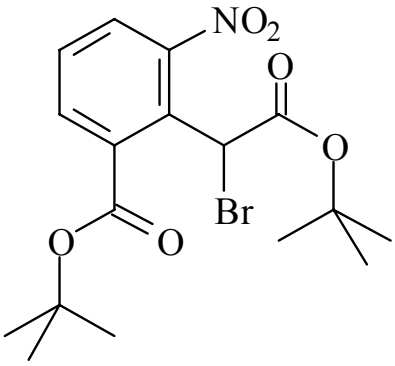


Figure 2: Whole-cell current of a EAAC1-expressed HEK 293 cells upon rapid application of 500 μM α-5-DCNB-D-aspartate. D-aspartate was released upon application of a 345 nm, 10 ns laser pulse (400 mJ/cm^2); the current was recorded at U = 0 mV (T = 22 °C, pH 7.4, V = 0 mV).

Appendix IV

α,6-DCNB



5d

Sample archive file for AM1 calculation:

MOPAC 93.00

C17 H22 N O6 Br

T=10000 AM1 EF PRECISE CHARGE=0 VECTORS

GEOMETRY OPTIMISED USING EIGENVECTOR FOLLOWING (EF).
SCF FIELD WAS ACHIEVED

HEAT OF FORMATION = -141.943572 KCAL = -593.89191 KJ
ELECTRONIC ENERGY = -37669.059341 EV
CORE-CORE REPULSION = 32709.754832 EV
DIPOLE = 5.93559 DEBYE SYMMETRY: C1
NO. OF FILLED LEVELS = 69
IONIZATION POTENTIAL = 10.559184 EV
HOMO LUMO ENERGIES (EV) = -10.559 -1.331
MOLECULAR WEIGHT = 416.268
SCF CALCULATIONS = 64
COMPUTATION TIME = 2 MINUTES AND 49.281 SECONDS

FINAL GEOMETRY OBTAINED

T=10000 AM1 EF PRECISE CHARGE=0 VECTORS

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 C    1.41282993  1    0.0000000  0    0.0000000  0    1    0    0   -0.1094
 C    1.40785841  1  121.5451502  1    0.0000000  0    2    1    0   -0.0600
 C    1.38872845  1  119.9741932  1   -1.1748554  1    3    2    1   -0.1350
 C    1.40977204  1  116.8984745  1   -0.7203953  1    1    2    3   -0.0842
 C    1.39907928  1  121.4319013  1    2.3825155  1    5    1    2   -0.0508
 H    1.10487049  1  120.0575577  1  179.7405398  1    3    2    4    0.1724
 H    1.10326451  1  120.3484981  1 -179.8760191  1    6    4    5    0.1627
 C    1.48611851  1  122.4826738  1 -178.2044381  1    5    1    6    0.3533
 C    1.49336708  1  122.1224812  1  179.4801741  1    1    2    5   -0.1648
Br    1.94594851  1  110.1247187  1 -118.0728834  1   10    1    2    0.0397
 N    1.49414654  1  123.1447792  1  178.9774274  1    2    1    3    0.5653
 O    1.20122864  1  120.2499685  1   39.1437231  1   12    2    1   -0.3539
 C    1.51455843  1  113.8026424  1  112.6664637  1   10    1    2    0.3317
 H    1.12724447  1  112.6258043  1  174.1004545  1   10    1    5    0.1883
 O    1.22819787  1  127.4297205  1   22.4931571  1   14   10   11   -0.3159
 O    1.36051431  1  111.0669818  1 -163.4934502  1   14   10   11   -0.2792
 O    1.20115727  1  121.9125378  1 -177.8904532  1   12   13    2   -0.3417
 C    1.45553345  1  120.2000955  1 -177.2184204  1   17   14   10    0.1063
 C    1.52705244  1  102.8306068  1 -174.3936721  1   19   17   14   -0.2113
 C    1.51881904  1  110.9081960  1  118.3151980  1   19   17   20   -0.2425
 C    1.51975378  1  109.8306960  1 -117.6659817  1   19   17   20   -0.2413
 O    1.36081470  1  111.8156560  1 -131.2443269  1    9    5    1   -0.2725
 O    1.23147883  1  120.9424888  1  175.9531972  1    9   23    5   -0.3344
 C    1.45470137  1  120.5877464  1 -178.7436640  1   23    9    5    0.1019
 C    1.52762402  1  102.7491016  1 -178.4437136  1   25   23    9   -0.2115
 C    1.51873693  1  110.8146938  1  117.9765829  1   25   23   26   -0.2424
 C    1.51947700  1  110.4282903  1 -117.8281972  1   25   23   26   -0.2414
 H    1.10089639  1  120.0331078  1  179.7225113  1    4    3    6    0.1569
 H    1.11524035  1  109.0110453  1 -175.1572638  1   20   19   17    0.0864
 H    1.11569942  1  110.2559586  1  119.9701611  1   20   19   30    0.0892
 H    1.11529452  1  110.1179733  1 -119.9764323  1   20   19   30    0.0857
 H    1.11709585  1  110.5391072  1 -165.6015570  1   21   19   20    0.1080
 H    1.11539112  1  109.6279990  1  120.3054829  1   21   19   33    0.0789
 H    1.11771895  1  109.9299274  1 -119.3746146  1   21   19   33    0.1055
 H    1.11569685  1  109.3201775  1  175.0546404  1   22   19   17    0.0861
 H    1.11694049  1  110.3670273  1  119.7816056  1   22   19   36    0.1058
 H    1.11595007  1  110.2495819  1 -119.8650015  1   22   19   36    0.0845
 H    1.11520788  1  108.9940241  1  179.7030770  1   26   25   23    0.0884
 H    1.11539571  1  110.2547369  1  120.0150856  1   26   25   39    0.0847
 H    1.11559712  1  110.1377885  1 -119.9791243  1   26   25   39    0.0914
 H    1.11585000  1  109.3423873  1 -169.9871741  1   27   25   23    0.0858
 H    1.11715583  1  108.9405068  1  121.0558051  1   27   42   25    0.1145
 H    1.11617545  1  109.0465547  1 -120.4119274  1   27   42   25    0.0896
 H    1.11591592  1  109.3113832  1  170.3013445  1   28   25   23    0.0880
 H    1.11589960  1  108.9622558  1  120.4799490  1   28   45   25    0.0807
 H    1.11689960  1  108.8891322  1 -120.8485068  1   28   45   25    0.1087
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