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Il Farmaco 58 (2003) 173–183
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Ligands for expression cloning and isolation of $GABA_B$ receptors

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Accepted 4 July 2002

Abstract

The scope of the plenary lecture at the occasion of the Xth Meeting on Heterocyclic Structures in Medicinal Chemistry, Palermo 2002, is considerably larger than that of the main lecture at the XVIth International Symposium on Medicinal Chemistry, Bologna 2000, described by Froestl et al. in *Farmaco* 56 (2001) 101. Additional information is presented, in particular, on the reaction conditions for the 31 step synthesis of the combined affinity chromatography and photoaffinity radioligand [^{125}I]CGP84963 and on the recent developments of the molecular biology of $GABA_B$ receptors.

Keywords: $GABA_B$ receptors 1a to 1g; $GABA_B$ receptor 2; Interplay between GB1 and GB2; Synthesis of [^{125}I]CGP84963

1. Historical Background

In 1950 Roberts and Frankel and the group of Awapara independently found that γ-aminobutyric acid (GABA, Fig. 1) is present in brain tissue [1,2]. In 1966 Krnjevic and Schwartz could show via electrophysiological studies that GABA acts as an inhibitory neurotransmitter [3]. GABA is synthesized in the brain via decarboxylation of L-glutamic acid by the enzyme glutamic acid decarboxylase (GAD), whereas, given orally, it is not capable to penetrate the blood–brain barrier. In 1962 the CIBA chemist Heinrich Keberle synthesized a lipophilic derivative of GABA, β-*p*-chlorophenyl-GABA, baclofen, which enters the CNS to a small extent sufficient to exert valuable muscle relaxant and analgesic properties [4]. This drug was marketed, as a racemate, in 1972 and is still widely prescribed for the treatment of spasticity in patients suffering from multiple sclerosis or hemi- and tetraplegia [5]. In 1980 Norman G. Bowery recognized that the (*R*)-(−)-enantiomer of baclofen (Fig. 1) stereoselectively interacts with a GABA receptor [6]. This interaction could not be blocked by the GABA antagonist bicuculline first introduced by Curtis et al. in 1970 [7]. Hence, the bicuculline insensitive receptor was designated as $GABA_B$ receptor, the bicuculline sensitive as $GABA_A$ receptor [8].

In 1987 Barnard et al. at Cambridge succeeded to clone the $GABA_A$ receptor, an ion channel coupled receptor, which on activation modulates fast synaptic activity via chloride ion influx into the cell [9]. Several groups of highly competent scientists attempted to elucidate the molecular structure of the G-protein coupled, or metabotropic, $GABA_B$ receptor. Two groups tried to isolate the receptor via protein purification from bovine (Kuriyama et al. [10]) or pig brain (Facklam and Bowery [11]). Much work was devoted to attempts of expression cloning in *Xenopus* oocytes, by Barnard in England, by the groups of Tanaka and Sekiguchi in Japan [12,13], by American (Woodward and Miledi [14], Lester, Olson) and German researchers (Seeburg). However, all attempts pursuing either approach ultimately failed.

2. A second class of selective $GABA_B$ receptor agonists

In 1984 chemists at the Central Research Laboratories of Ciba–Geigy in Manchester discovered a novel class of $GABA_B$ receptor ligands. They replaced the

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doi:10.1016/S0014-827X(03)00018-1

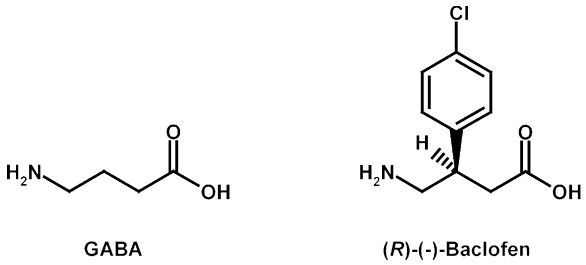


Fig. 1.

carboxylic acid group of all natural aminoacids by the bioisosteric phosphinic acid moiety [15,16]. None of the phosphinic acid analogues of α-aminoacids showed any interesting biological activity. On the contrary, the phosphinic acid analogue of GABA, CGP27492 (Fig. 2), showed a very high affinity towards $GABA_B$ receptors exceeding significantly the affinities of both GABA and baclofen (IC_{50} values of 2, 25 and 35 nM, respectively, for the inhibition of binding of [^{3}H]baclofen to $GABA_B$ receptors on cat cerebellum membranes) [17]. CGP27492 interacted with $GABA_A$ receptors with 4500 times lower affinity. Even today this compound is the most potent $GABA_B$ receptor agonist. [^{3}H]CGP-27492 has replaced [^{3}H]baclofen as radioligand due to its higher potency, its high specific binding and due to the possibility of carrying out filtration assays [18,19]. Also the methyl homologue CGP35024 (Fig. 2), identical with SK&F97541, synthesized simultaneously by Smith Kline & French chemists, is a potent $GABA_B$ receptor agonist ($IC_{50} = 7$ nM) [17,20].

3. Discovery of $GABA_B$ receptor antagonists

In 1987, 25 years after the synthesis of baclofen, it was found that phosphinic acid analogues with substituents larger than methyl display properties of low affinity $GABA_B$ receptor antagonists (CGP's 36 216, 35 348 and 36 742 with IC_{50} values of 2, 27 and 38 μM, respectively, for the inhibition of binding of [^{3}H]CGP27492 to $GABA_B$ receptors on rat cortex membranes; Fig. 2) [21]. Compounds with substituents in size between methyl and ethyl, such as difluoromethyl (CGP47656, $IC_{50} = 89$ nM), display properties of partial $GABA_B$ receptor agonists [17]. Also the phosphonic acid analogue of baclofen, phaclofen, synthesized simultaneously in the laboratories of Rolf H. Prager in Adelaide and at Ciba–Geigy in Manchester, is a low affinity $GABA_B$ receptor antagonist (racemic phaclofen: $IC_{50} = 130$ μM; Fig. 2) [22].

Four years of optimization work of four chemistry laboratories were required to significantly improve the potency of this class of $GABA_B$ receptor antagonists in order to obtain compounds with single digit nanomolar affinity. The decisive breakthrough was achieved by Stuart J. Mickel, when he substituted the amino-group of the γ-aminopropyl-phosphinic acids with branched benzyl-substituents [23,24]. Most potent compounds are the 3,4-dichlorobenzyl derivative with an α-methyl substituent in (*S*)-configuration CGP54626A ($IC_{50} =$ 2.2 nM; inhibition of binding of [^{125}I]CGP64213 to $GABA_B$ receptors on rat cortex membranes, Fig. 3) and the *meta* carboxy-substituted benzyl derivative with an α-methyl substituent in (*R*)-configuration CGP62349 ($IC_{50} = 0.7$ nM; Fig. 3). It is not understood, why the α-methyl substituents are oriented in opposite stereochemical alignments in the 3,4-dichlorobenzyl and the 3-carboxybenzyl-substituted ligands for the high affinity diastereoisomers, respectively.

Both compounds are also available as tritiated radioligands [25,26]. In particular, [^{3}H]CGP62349 with a specific radioactivity of 85 Ci/mmol is used frequently for autoradiographic studies [27]. [^{11}C]CGP62349 was evaluated as a potential PET ligand at the Ospedale San Raffaele in Milan in the laboratories of Ferruccio Fazio [28,29].

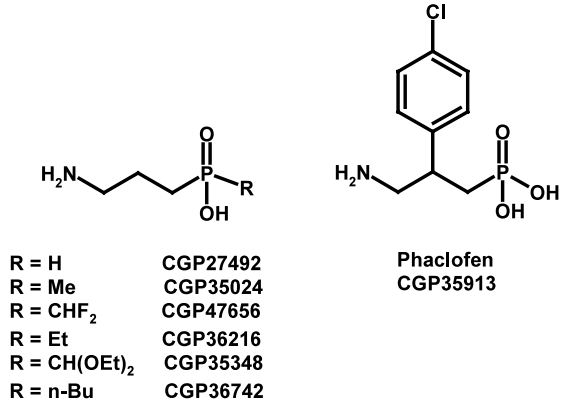


Fig. 2.

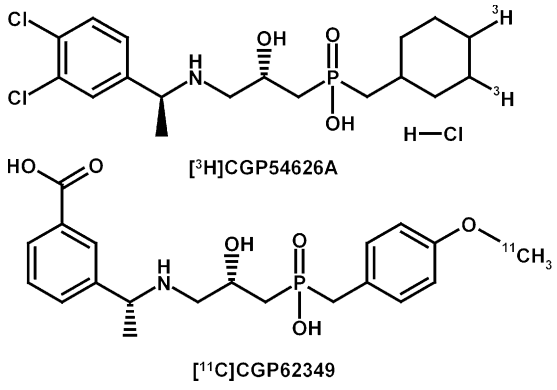


Fig. 3.

4. Ligands for expression cloning of $GABA_B$ receptors

A ligand for expression cloning must fulfil three requirements. We need:

- an antagonist labeling all affinity states of the receptor
- with very high affinity to the receptor ($IC_{50} < 5$ nM),
- which contains a radioisotope with very high specific radioactivity as ^{125}I or ^{32}P. In 1991 we synthesized CGP54748A ($IC_{50} = 5$ nM; Fig. 4), which we thought would fulfill the above mentioned criteria. However, during the exchange reaction with $Na^{125}I$ Amersham chemists reported complete decomposition of the product.

We first formulated a radical cleavage reaction for this decomposition (Fig. 5). However, as CGP57076A ($IC_{50} = 19$ nM; Fig. 4), without the 2-hydroxy substituent, also decomposed under same reaction conditions, fragmentation according to the mechanism outlined in Fig. 5 appears unlikely. Fragmentation of an intermediate benzylic radical seems to be more probable (Fig. 6). However, no further efforts were made to elucidate the precise mechanism of this reaction.

At this point we abandoned the attempts to prepare ^{125}I radioligands (maximal specific radioactivity: 2000 Ci/mmol) and we turned to other radioligands with less specific radioactivity, such as ^{35}S (maximal specific radioactivity: 1300 Ci/mmol). ^{35}S-labelled methionine derivatives are commercially available. We prepared novel phosphinic acid analogues containing a primary amino-group, to which the radiolabelled aminoacids were attached via peptide bonds.

The ε-aminopentyl phosphinic acid CGP57604A (Fig. 7) proved to be useful. It shows only moderate affinity to $GABA_B$ receptors ($IC_{50} = 110$ nM). However, after acylation, high affinity $GABA_B$ receptor antagonists such as CGP's 80 936 or 63 776 were obtained ($IC_{50} = 13$ nM for both; Fig. 7). The condensation of CGP57604A with Boc-protected methionine led to CGP61302, and

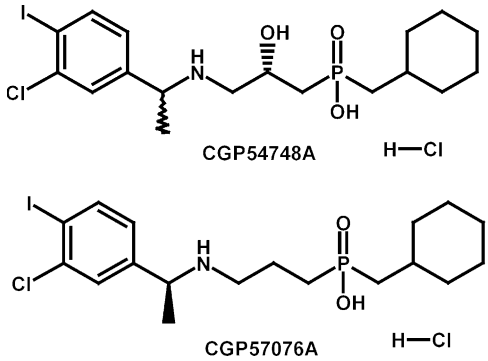


Fig. 4.

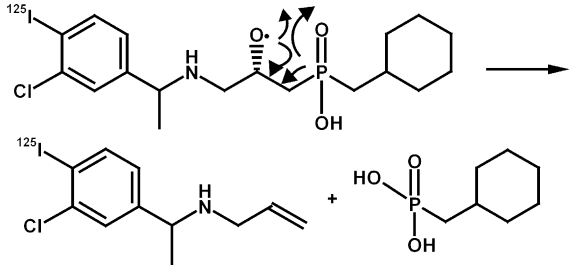


Fig. 5.

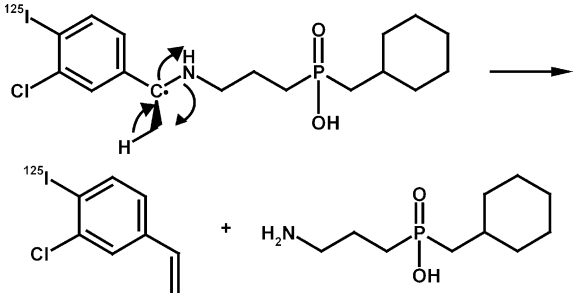


Fig. 6.

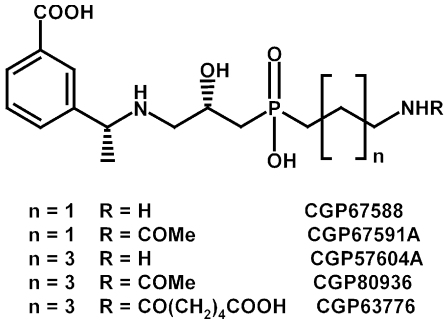


Fig. 7.

after deprotection, to CGP61857 with IC_{50} values of 31 and 26 nM, respectively (Fig. 8), which is not sufficient to be useful for expression cloning.

After all these negative results we decided to completely abandon radioactive ligands and to prepare fluorescent ligands instead, with the help of which one may also be capable to visualize transfected $GABA_B$ receptors. We condensed the ε-aminopentyl phosphinic acid CGP57604A (Fig. 7) with fluorescein–isothiocyanate to obtain CGP63626 ($IC_{50} = 123$ nM; Fig. 9), a compound with unacceptable affinity.

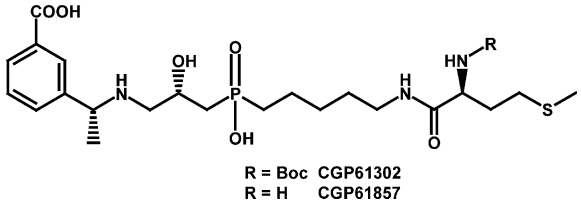


Fig. 8.

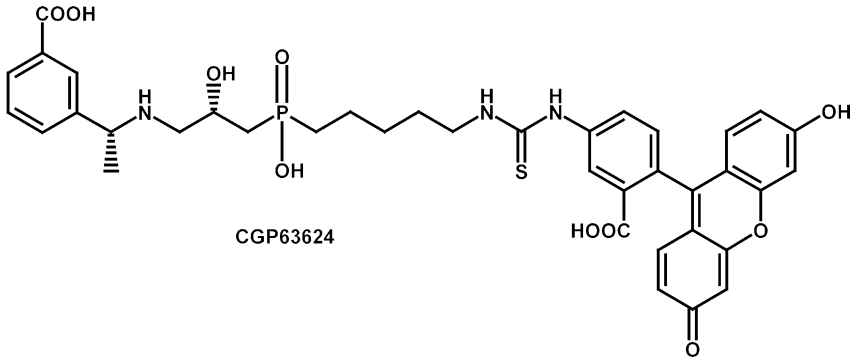


Fig. 9.

At this point we were very close to give up. In a last attempt we returned to fragmentation scheme outlined in Fig. 6 and tried to differentiate between inter- and intra-molecular fragmentation processes. If the strong radiation of the ^{125}I isotope cleaves the benzylic carbon–hydrogen bond of a neighboring molecule in an inter-molecular process, we cannot influence the decomposition. But, if the fragmentation occurs due to the proximity of the ^{125}I isotope to the fragile tertiary benzylic carbon–hydrogen bond in an intra-molecular process, we can place the radioactive iodine isotope as far away from this bond as possible. This reasoning finally led to the synthesis of [^{125}I]CGP64213 (Fig. 10) in July 1994, a compound with very high affinity and specific radioactivity, which was stable for one half-life of the ^{125}I isotope, i.e. 60 days [30].

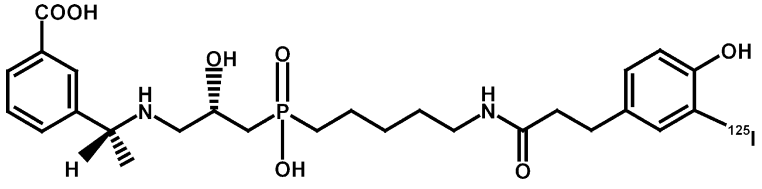
In late 1994 the molecular biologists Bettler and Kaupmann began to set up a screening procedure for [^{125}I]CGP64213 binding at transfected COS cells. They started with a commercial rat brain cDNA library containing 1.5 million clones, which were divided into pools of 2000 cDNA clones each and transfected into COS cells. Within 9 months they screened a total of 720 000 clones.

By introducing the photolabile azide group we obtained the photoaffinity radioligand [^{125}I]CGP71872 (Fig. 11) in July 1995, which on irradiation with a 24 W lamp of wavelength 365 nm for 3 min bound irreversibly to native $GABA_B$ receptors in rat cortex, cerebellum and spinal cord membranes revealing two bands of 130 and 100 K in SDS gel electrophoresis allowing, for the first time, the determination of the molecular weights of two $GABA_B$ receptor splice variants [30,31].

This was crucial information, as it showed that the cDNA clone for the larger $GABA_B$ receptor splice variant must have an insert size of more than 4 kb, whereas the insert sizes of the clones of the commercial cDNA library were, at best, 3 kb. Therefore, a new cDNA library of two million clones was prepared from rat brain of postnatal day 7 and after screening of another 620 000 clones, the clones encoding $GABA_B$ receptor 1a, a protein of 960 amino acids and of $GABA_B$ receptor 1b, a protein of 844 amino acids, both with the typical seven transmembrane spanning domains of G-protein coupled receptors, were identified. These results were published in *Nature* in March 1997 [32], more than 17 years after the first pharmacological characterization of $GABA_B$ receptors by Bowery [6].

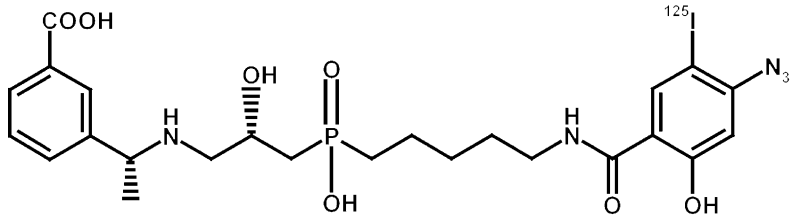
5. Ligands for the isolation of extracellular domains of $GABA_B$ receptors

In 1999 Malitschek et al. succeeded to express the soluble N-terminal extracellular domains (ECDs) of $GABA_B$ receptors 1a and 1b in insect cells using recombinant baculovirus. They showed that the photoaffinity ligand [^{125}I]CGP71872 binds to both receptor fragments and this binding can be blocked by prior exposure of the proteins to the potent $GABA_B$ receptor antagonist CGP54626A indicating that the N-terminal extracellular domains of both $GABA_B$ receptors 1a and 1b can fold correctly even when they are dissociated



[^{125}I]CGP64213 K_D = 1.2 nM, specific radioactivity >2000 Ci/mmol

Fig. 10.



[^{125}I]CGP71872 K_D = 1 nM, specific radioactivity >2000 Ci/mmol

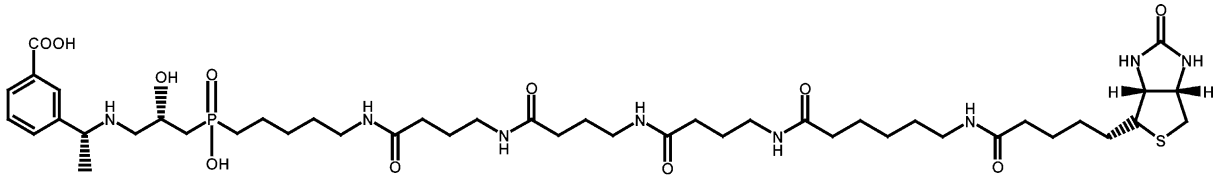
Fig. 11.

from the transmembrane domains [33]. Now we can attempt to isolate the soluble N-terminal extracellular domains of $GABA_B$ receptor 1a or 1b, purify these proteins and crystallize them. The X-ray structure will, finally, provide precise data on the three dimensional environment of the binding site of $GABA_B$ receptor 1.

A very efficient method of protein purification is affinity chromatography using ligands to which biotin is attached with its extremely high affinity to avidin ($K_D = 10^{-15}$ M). We estimated that we would need a spacer between the $GABA_B$ receptor binding part of the ligand and biotin of a length of approximately 24 Å. We calculated that the binding region of a 'classical' G-protein coupled receptor is located at a depth of 15 Å and that the binding pocket of biotin in avidin is located at a depth of 9 Å. One example for such a ligand is CGP73415 ($IC_{50} = 33$ nM; Fig. 12), with 22 bonds between the $GABA_B$ receptor binding part and biotin, for which experiments using [^{35}S]streptavidin proved that the ligand interacted simultaneously with native $GABA_B$ receptors from rat cerebral membranes and streptavidin [34].

CGP73415 may retain the ECD of $GABA_B$ receptor 1 on avidin columns. Desorption of the receptor fragment after purification may be achieved by competition with potent antagonists, such as CGP54626A (Fig. 3).

But, due to the equilibrium between association and dissociation of the ligand to the receptor binding site we



CGP73415

Fig. 12.

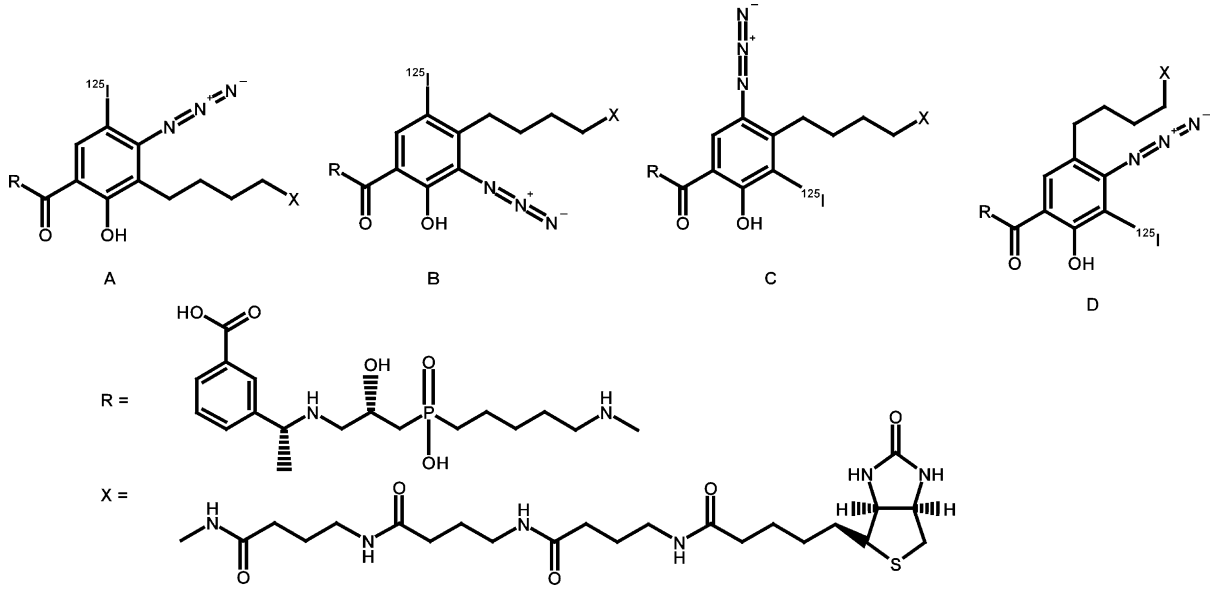
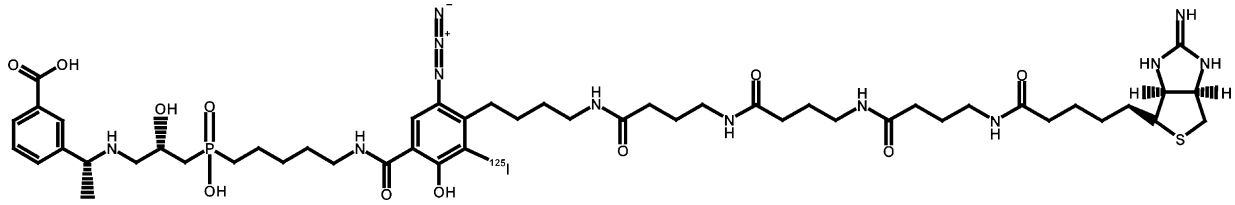


Fig. 13.

will obtain, in any case, a mixture of three species, the $GABA_B$ receptor fragment with and without the ligand plus unbound ligand. We thought that we may have a higher chance to induce crystallization in a solution of a single species than from a mixture of three components.

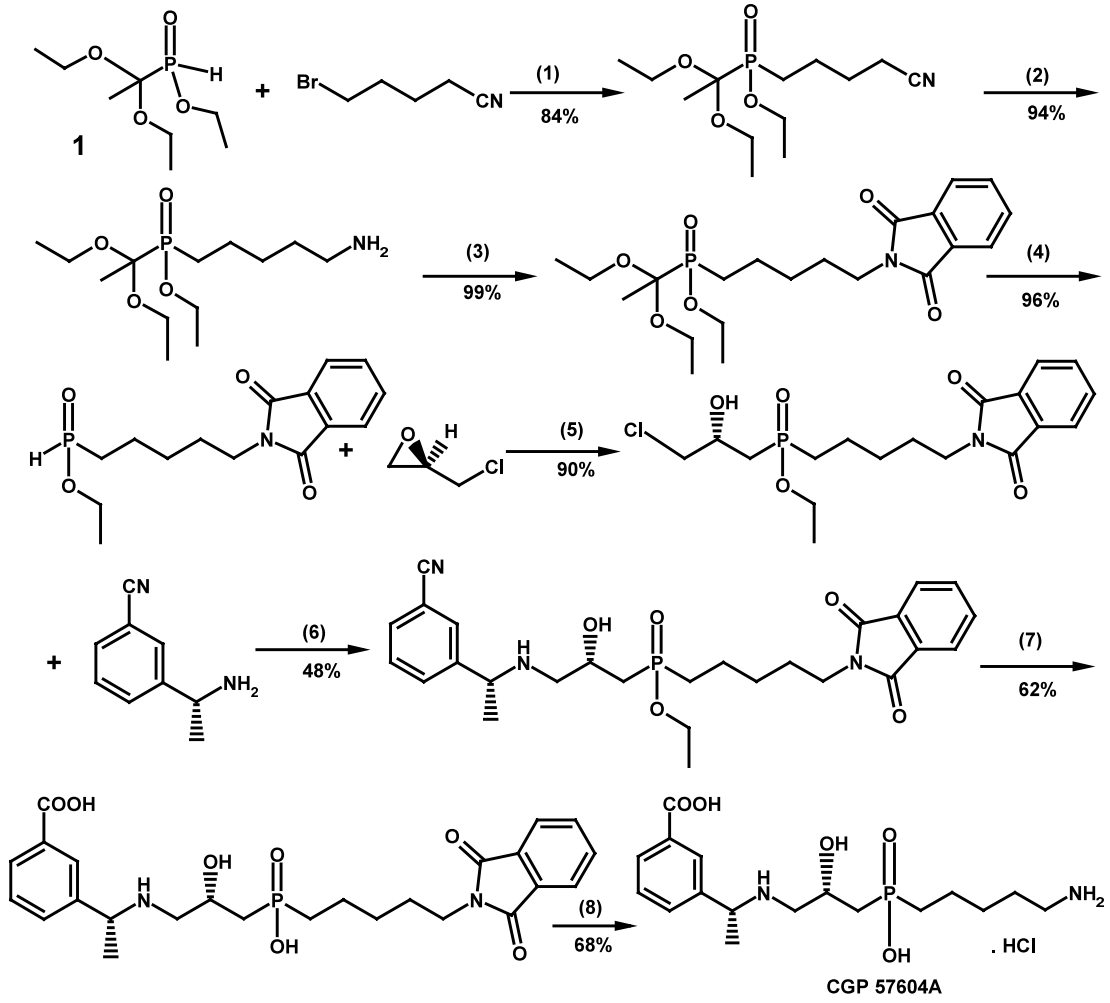
We designed a ligand combining in one molecule a photoaffinity and an affinity chromatography moiety. The most straightforward approach is to attach a spacer plus biotin as fifth substituent on the azidosalicylic acid moiety of the photoaffinity radioligand [^{125}I]CGP71872 (Fig. 11).

Theoretically we can envisage four chemically feasible permutations of the penta-substituted iodo azido salicylic acid as the OH-group directs the iodine substituent into *para* (A and B) or *ortho* positions (C and D), respectively (Fig. 13). Reactive groups at the receptor fragment bind to the nitrene intermediate generated by the photoreaction from the azide. In structures A, B or



[^{125}I]CGP84963

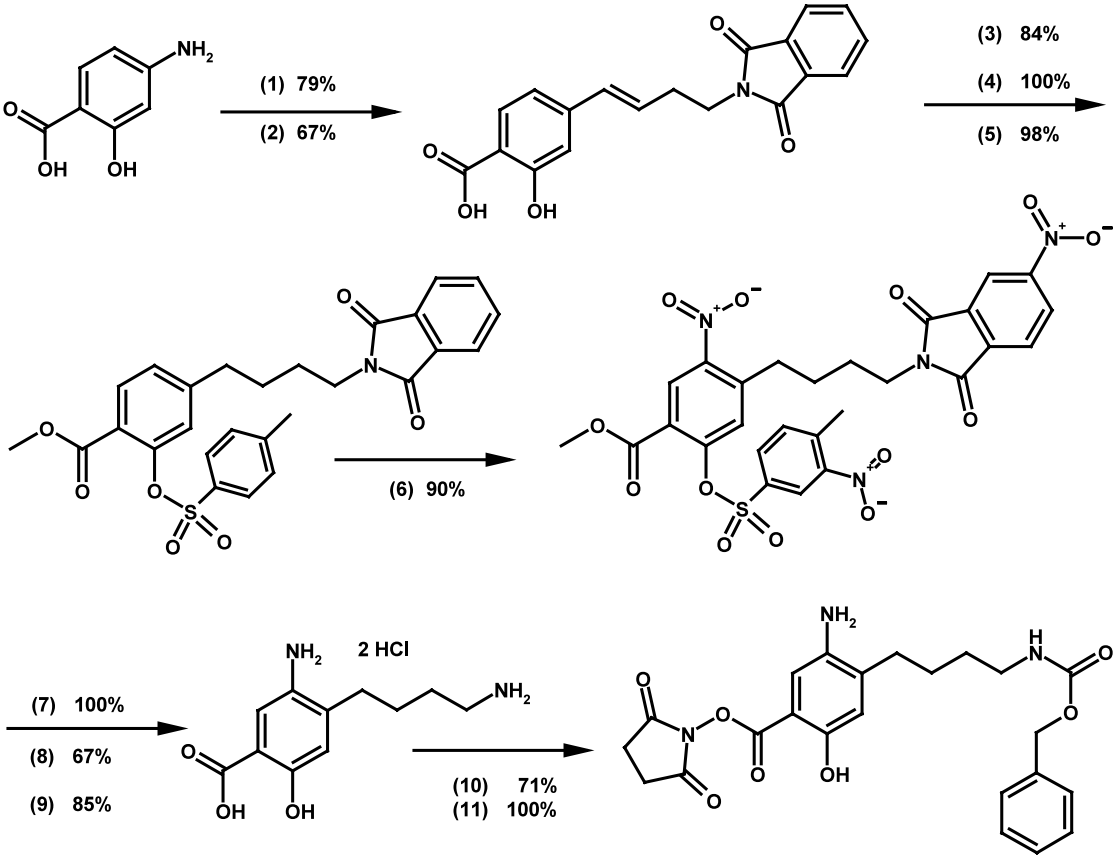
Fig. 14.



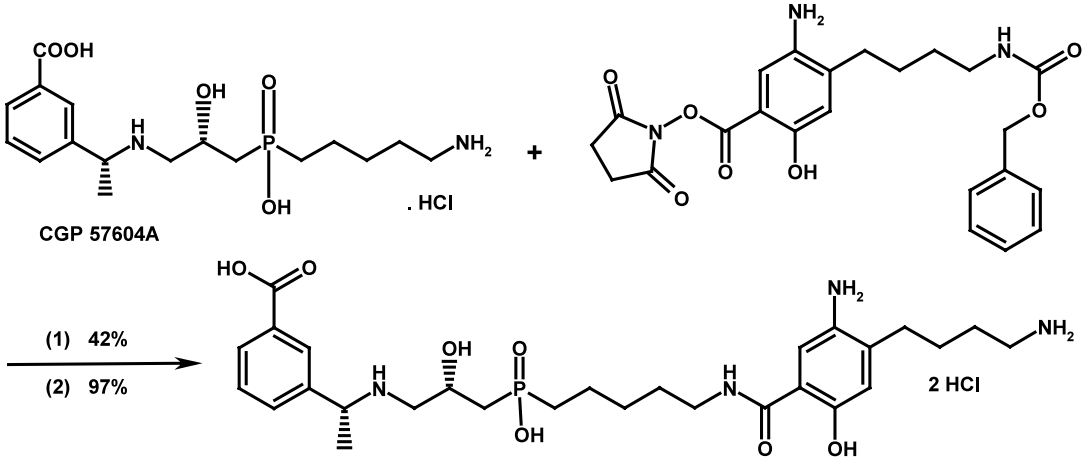
Scheme 1. Reagents and conditions: (1) NaH, THF, r.t., 3 h; 5-bromovaleronitril, r.t., 16 h; (2) Raney nickel, 4% NH_3 in EtOH, 45 °C, 16 h; (3) *N*-ethoxycarbonyl-phtalimid, Na_2CO_3, H_2O, CH_2Cl_2, r.t., 5 h; (4) Me_3SiCl, EtOH, CH_2Cl_2 (1:9), r.t., 17 h; (5) Me_3SiCl, Et_3N, THF, r.t., 17 h; (*R*)-epichlorohydrin, 10 mol.% $ZnCl_2$, THF, 80 °C, 17 h; HOAc, MeOH, r.t., 17 h; (6) *i*-Pr_2EtN, EtOH, 80 °C, 7 days; (7) LiOH, EtOH, H_2O (1:1), 100 °C, 17 h; MeOH, H_3PO_4; (8) conc. HCl, 100 °C, 17 h.

D both *ortho* positions adjacent to the azido group are substituted, only in structure C one *ortho* position is free causing less steric hindrance for the reaction with the protein than structures A, B, or D.

However, by attaching the ligand of structure C irreversibly to the $GABA_B$ receptor fragment we have to face another serious problem, i.e. to detach the receptor–ligand complex after the purification step from the avidin columns. A viable solution to this problem is the use of 2-imino–biotin, whose affinity to avidin is pH-dependent. Protonated 2-iminium–biotin binds to avidin with eight orders of magnitude weaker affinity ($K_D = 10^{-3}$ M) than 2-imino–biotin ($K_D = 3.5 \times 10^{-11}$ M). Recovery of the protein–ligand complex from avidin columns can be achieved by washing with ammonium acetate solutions at pH 4 [35].



Scheme 2. Reagents and conditions: (1) $NaNO_2$, H_2SO_4, 0–5 °C, I_2, NaI, H_2O, 0–5 °C, 6 h, r.t., 18 h; (2) *N*-3-butenyl-phtalimide, $Pd(OAc)_2$, P(*o*-tolyl)$_3$, CH_3CN, Et_3N, 70 °C, 96 h; (3) MeOH, H_2SO_4, reflux, 16 h; (4) H_2, Pd/C, EtOAc, r.t., 1 bar, 30 min; (5) TosCl, K_2CO_3, acetone, reflux, 16 h; (6) 100% HNO_3, 0–5 °C, 3 h, r.t., 16 h; (7) 1 N NaOH, 80 °C, 4 h; (8) $N_2H_4 \cdot H_2O$, EtOH, reflux, 16 h, 0.1 N HCl; (9) H_2, PtO_2, MeOH, HCl, r.t., 1 bar, 90 min; (10) *N*-hydroxysuccinimidyl-benzylcarbonate, $NaHCO_3$, acetone, H_2O, r.t., 16 h; (11) *N*-hydroxysuccinimide, DCC, dioxane, r.t., 16 h.



Scheme 3. Reagents and conditions: (1) *i*-Pr_2EtN, DMF, r.t., 94 h; (2) H_2, Pd/C, MeOH, HCl, r.t., 1 bar, 2 h.

It required 31 steps to synthesize [^{125}I]CGP84963 (Fig. 14), for which a dissociation constant K_D of 2 nM was calculated from best-fit analyses of saturation curves. Thirty percent of the total radioactivity was bound irreversibly to native $GABA_B$ receptors of rat cortex after the photoreaction [34].

The synthesis of combined photoaffinity and affinity chromatography radioligand [^{125}I]CGP84963 is outlined in the following five reaction Schemes starting from cheap inorganic hypophosphorus acid, which is condensed with triethylorthoacetate and boron trifluoride etherate to give ethyl(1,1-diethoxyethyl)-phosphinate (**1**) (Scheme 1) in 60% yield [17]. Scheme 1 shows the eight step synthesis of the ε-aminopentyl phosphinic acid CGP57604A, which contains the $GABA_B$ receptor binding part. Scheme 2 shows the 11 step synthesis of the photoaffinity moiety of the ligand, Scheme 3 the condensation of the $GABA_B$ receptor binding part and the photoaffinity part (two steps), Scheme 4 the synthesis of the spacer consisting of three molecules of GABA (five steps). Scheme 5 illustrates the last five steps to [^{125}I]CGP84963.

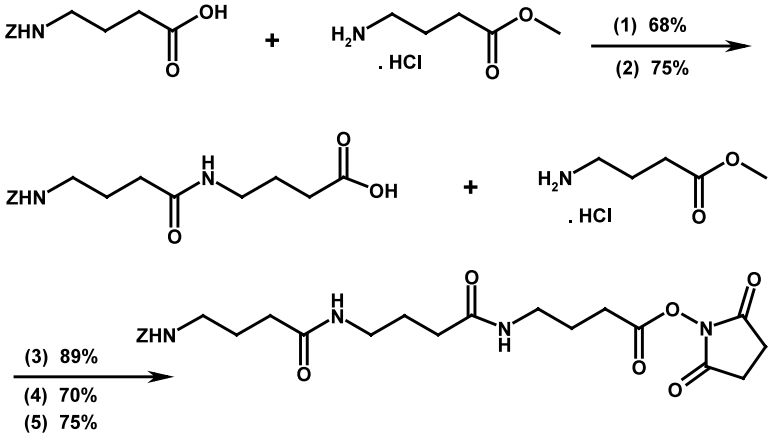
Experiments are currently underway in the laboratories of Professor Markus Grütter at the University of Zurich to purify the extracellular domain of $GABA_B$ receptor 1a expressed in large quantities in *Escherichia coli* using this ligand with the aim of obtaining a crystalline receptor fragment–ligand complex suitable for X-ray structure determination.

6. Update on most recent developments and outlook

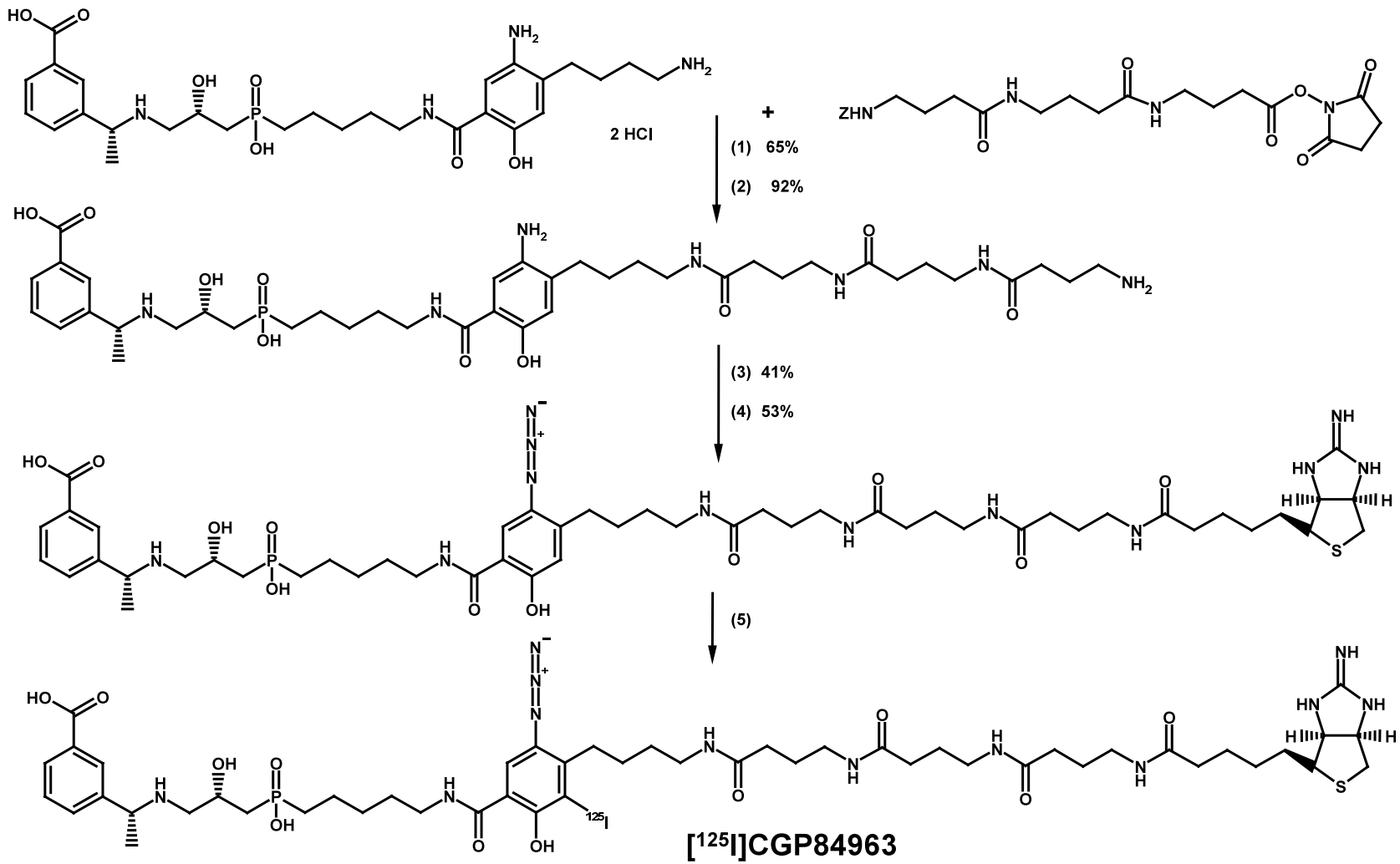
In late 1998 the $GABA_B$ receptor 2, a protein of 941 aminoacids, was cloned simultaneously by four groups, at Novartis, Glaxo, Synaptic Pharmaceuticals and BASF [36–39]. In order to get functionally active receptors $GABA_B$ receptors 1 and 2 have to form heterodimers via a coiled-coil interaction, a stretch of 23 aminoacids at their cytoplasmic C-termini. This was the first example of a heterodimeric G-protein coupled receptor. Jean-Philippe Pin and Roberto Pellicciari showed independently that the ligands bind via a Venus Flytrap module [40–43]. GB1 binds GABA and all known $GABA_B$ receptor ligands. GB2 does not bind GABA or another endogenous ligand. According to current knowledge GB2 has three tasks: first to move GB1 to the cell surface by masking an intracellular retention signal [44–46], second, to increase the affinity of agonist binding to GB1 [36,47] and third, to effect G-protein activation via a distinct hydrophobic residue in its third intracellular loop [47–50]. This makes the $GABA_B$ receptor the only GPCR, in which the agonist binding site and the G-protein coupling domain are located in two different subunits. The question is, of course, how the signal is translocated from one subunit to the other.

This is, however, not the only unresolved question in $GABA_B$ receptor research. The most important one—from the viewpoint of drug research—is the issue of $GABA_B$ receptor subtypes, which were proposed after numerous pharmacological [51,52] and electrophysiological studies [53,54]. One of the major functions of $GABA_B$ receptors is the modulation of the release of a multitude of neurotransmitters, such as glutamate, dopamine, noradrenaline, serotonin, acetylcholine substance P, cholecystokinin and somatostatin via presynaptic $GABA_B$ heteroreceptors, and of GABA via the proposed presynaptic autoreceptor. It would be highly attractive to selectively target each individual subtype. But, it appears that the GB1 plus GB2 heterodimer is the only $GABA_B$ receptor in the brain, since the knockout of the pan GB1a & 1b subunit is sufficient to suppress all $GABA_B$ mediated effects in vivo [55,56].

We do not know about the physiological importance of different combinations of $GABA_B$ receptor subunits to form distinct heterodimers. So far at least seven isoforms of GB1, 1a–1g (1c [57], 1c and 1d [58], 1e [59], 1f [60], 1g [61]) have been identified. Three isoforms of



Scheme 4. Reagents and conditions: (1) DCC, Et_3N, CH_2Cl_2, r.t., 16 h; (2) 2 N NaOH, MeOH, r.t., 16 h; (3) DCC, Et_3N, CH_2Cl_2, r.t., 16 h; (4) 2 N NaOH, MeOH, r.t., 16 h; (5) *N*-hydroxy-succinimide, DCC, dioxane, r.t., 65 h.



Scheme 5. Reagents and conditions: (1) *i*-Pr_2EtN, DMF, r.t., 65 h; (2) H_2, Pd/C, MeOH, HCl, r.t., 1 bar, 3.5 h; (3) *N*-hydroxysuccinimido-2-iminobiotin hydrobromide (Pierce), *i*-Pr_2EtN, DMF, r.t., 65 h; (4) $NaNO_2$, 80% HOAc, −15 °C, 10 min, NaN_3, H_2O, −15 °C to 0–5 °C, 72 h; (5) $Na^{125}I$, phosphate buffer, pH 7.4, H_2O_2, catalytic amounts lactoperoxidase, 0–5 °C, 6 h.

GB2, 2a–2c have been described [62], but their existence was recently questioned [63].

Likewise, we do not know about the differences of $GABA_B$ receptor subunit expression in normal and in pathological tissues in diseases such as spasticity, epilepsy, chronic pain, esophageal reflux, and the various forms of drug abuse, for all of which $GABA_B$ receptor ligands may provide valuable medications.

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