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Benzodiazepines, the Story of the Antagonist Flumazenil and of the Partial Agonist Bretazenil

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Abstract. The story of flumazenil and bretazenil is a typical example of a serendipitious drug discovery. In 1979 benzodiazepine antagonists were unknown. No one was looking for them, but they were discovered nevertheless. *Ro 15-1788* was selected for clinical trial. Today this compound has the generic name flumazenil, the trade name *Anexate*, and is registered in 70 countries. It is the first specific benzodiazepine receptor antagonist for clinical use. Minor structural modifications of benzodiazepine antagonists led to partial agonists, compounds with powerful anxiolytic and anticonvulsant properties. Compared with full agonists they have markedly reduced sedative and alcohol-potentiating effects. Bretazenil is in clinical trial; it exhibits promising anxiolytic activity especially in patients with panic attacks. In open clinical studies it has shown remarkable antipsychotic activity. Structure-activity relationships and spin-offs are discussed.

1. The Chemical Pedigree of Flumazenil (20)

Chlordiazepoxide (**1**, *Librium*) is the first anxiolytic with the structure of a benzodiazepine. It was synthesized in 1955 and submitted for testing by *Leo H. Sternbach* in 1957 at *F. Hoffmann-La Roche*, Nutley, USA [1]. The time elapsing between the first pharmacological testing and introduction on the market was only two and a half years. In 1963, diazepam (**2**, *Valium*), the best known and best investigated benzodiazepine reached the market place.

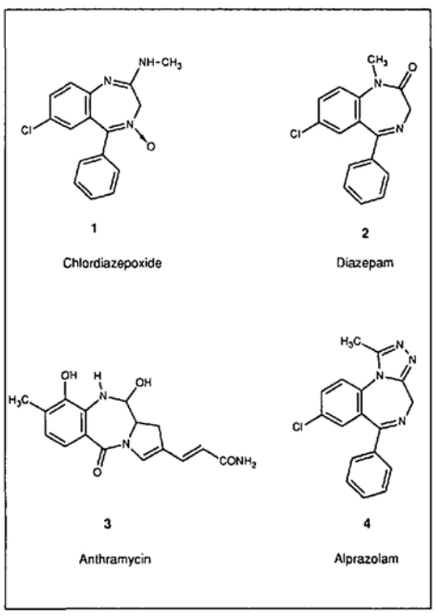
At this time, when benzodiazepines were a major focus of attention in Nutley, one group there, *Leimgruber*, *Batcho*, and *Schenker* was working on antibiotics. They isolated anthramycin (**3**) and elucidated its structure [2]. To their great surprise, they discovered that it, too, was a 1,4-benzodiazepine.

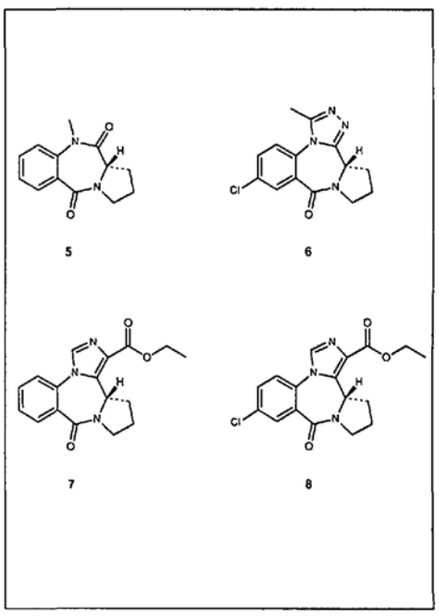
Anthramycin (**3**) was later found to have no affinity for the benzodiazepine receptor (BZR). Chemists working at *Lederle* at that time were not in a position to know this, when they started synthesizing simpler analogues of anthramycin, since the existence of the BZR had not yet been established. A number of their compounds showed some anticonvulsant and anti-conflict activity in animal tests. Thus, **5** was selected as a candidate for anxiolytic evaluation in a limited clinical trial in man, but it failed to produce sufficient anxiolytic activity. Their results were published [3].

This is one branch of the pedigree; the other stems from the triazolobenzo-diazepines, which were synthesized and investigated at about the same time at *Takeda Chemical Industries* in Japan and at the *Upjohn Company* in the United States [4][5]. These are more potent than previous benzodiazepines.

Alprazolam (**4**), to take one example, is more potent in terms of anticonvulsant and anxiolytic effects. The question that now arose was whether annelation by a triazolo ring would also work in a different series, *e.g.*, with the *Lederle* anthramycin derivative? Would this result in a more potent substance? Compound **6** was synthesized to answer these questions:

In December 1978, the two imida-





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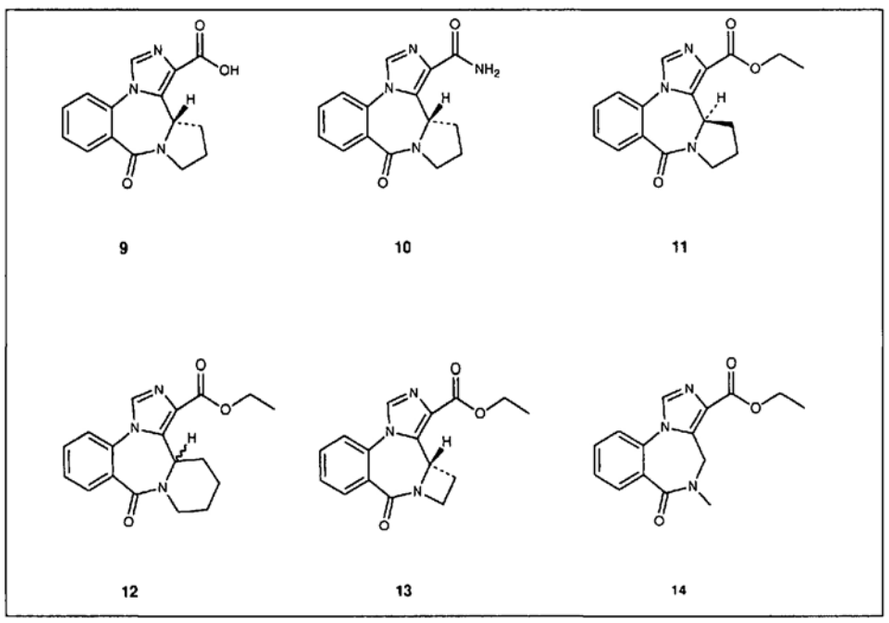
zoesters **7** and **8** were synthesized and submitted for testing together with the triazolo compound **6**. All turned out to be inactive in the usual tests for benzodiazepines.

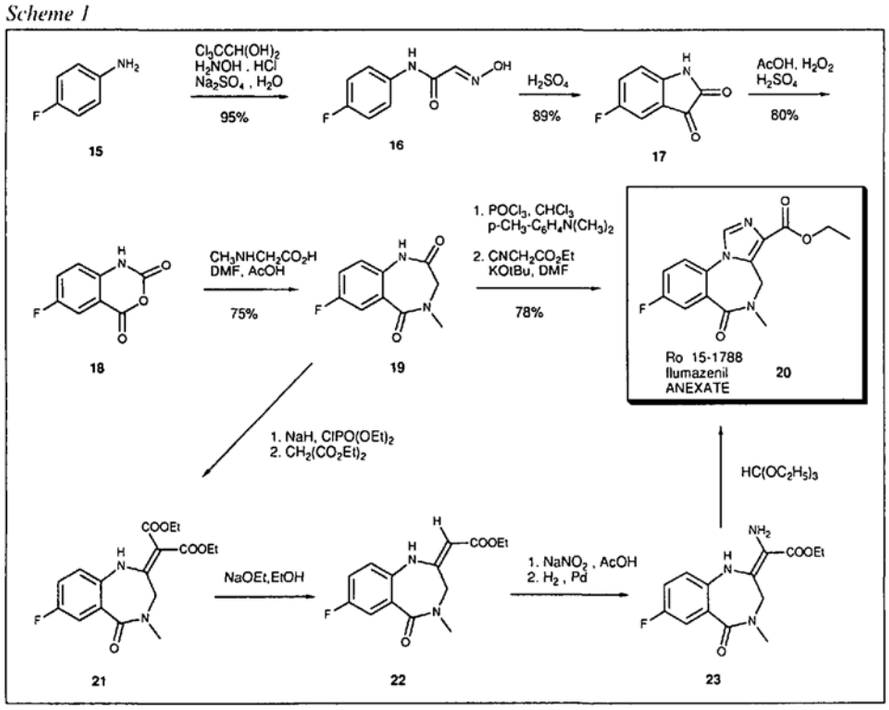
Luckily for us, the benzodiazepine receptor (BZR) had been discovered the year before [6][7]. Using the ^{3}H-diazepam binding assay it became possible to determine the affinity of a compound for the BZR *in vitro*. The three compounds were tested in this assay, and the triazolo derivative **6** that we had favored proved to have no affinity for the BZR. However, the imidazo-ester **8**, with a Cl-atom in the same position as in diazepam, had an IC_{50} of 62 nM. Compound **7**, without the chlorine, had with 6.4 nM an even greater affinity for the BZR than diazepam. As a consequence of this preliminary result, compounds without the Cl-atom in the phenyl ring were synthesized. Thus, the first three compounds in this new series already indicated that structure-activity relationship were different from those determined for the older benzodiazepines, such as **2**. The fact that **7** and **8**, with an affinity for the BZR, failed to show BZ-like activity was not very discouraging initially, because for metabolic or pharmacokinetic reasons *in vitro* activity is often not accompanied by *in vivo* activity. Therefore, synthetic work in the series of imidazobenzodiazepinones continued.

Table 1. *Binding Activities of Compounds Synthesized on the Way to Flumazenil* (**20**)

Compound	Inhibition of ^3H-diazepam binding *in vitro*, IC_{50}, in nM
9	> 1000
10	> 1000
11	> 1000
12	> 1000
13	1.3
14	3.0



Scheme 1



Rapid hydrolysis of the ester **7**, the lead compound, to the inactive acid **9** was a possible explanation for inactivity of **7** *in vivo*. The amide **10** was also inactive *in vitro*. This was surprising since the classical benzodiazepine series contains a number of highly potent imidazo amides. The lead compound **7** is chiral and has the absolute configuration (*S*). The (*R*)-enantiomer **11** is inactive in the BZR binding test. Compound **12**, containing the six-membered ring, was inactive but the result with the *N*-methyl compound **14** showed that this ring is not required for increased affinity for the BZR. Later on, **13** with the four-membered ring was synthesized; it has a high affinity for the BZR (*Table 1*).

A considerable number of compounds synthesized in the first half of 1979 had very high affinity for the BZR. In spite of this, the compounds displayed no or only borderline activity *in vivo*. The possibility that these compounds might be antagonists was discussed. Our suspicions were increased, when further testing revealed that the lead compound **7** also bound to the receptor *in vivo*, demonstrating that the target, the BZR in the brain, was reached. In early September 1979 a rat was sedated with diazepam (**2**), after which it received an injection of **7**. Even before the injection was finished the rat got up and walked off. New tests were immediately devised. In each situation the effects of diazepam and other benzodiazepines were reversed by **7**.

2. Optimization: Flumazenil (20) (*Scheme 1*)

The lead compound **7** was not a pure antagonist. Some minor agonistic activity was found, *e.g.* in a conflict test in rats. We wanted a pure antagonist for therapeutic applications and as a scientific tool. In October 1979 *Ro 15-1788*, flumazenil (**20**)

was synthesized, and, in January 1980, selected for clinical trial.

2.1. *The Synthesis of Flumazenil* (20)

Starting with 4-fluoroaniline (**15**) the isatin **17** is synthesized *via* the *Sandmeyer* synthesis; isatin is then oxidized with peracetic acid to the isatoic anhydride **18**. Reaction with sarcosine in DMF leads to the benzodiazepine-2,5-dione **19**. This is converted to the iminochloride by reaction with $POCl_3$. In the key step the imidazoester is built up by reaction with deprotonated ethyl isocyanoacetate [8]. Since ethyl isocyanoacetate is not very stable, an alternative synthesis based on the synthesis of midazolam was developed for large scale-production. In this synthesis diethylmalonate is used. The diester **21** is then transformed to the monoester **22** by deethoxycarbonylation. Nitrosation and catalytic reduction lead to the amino compound **23**. The final carbon atom is introduced by reaction with the orthoester. In 1981 our paper on the first selective antagonist of benzodiazepines [9] caused enormous interest in flumazenil in the scientific community. In 1987, flumazenil was launched in Switzerland and France as *Anexate*. In 1988 *Roche* was awarded the prestigious Prix Galien in recognition of the therapeutic originality of *Anexate*. In the meantime, it has been registered in 70 countries. The tradename in most countries is *Anexate*, in the United States *Romazicon*.

2.2. *Indications for Flumazenil* (20)

Flumazenil (**20**) is used when the effects of benzodiazepines are no longer desired. For example:

- To reverse the effect of benzodiazepine overdose.
- To reverse sedation in patients who have been given benzodiazepines to help them tolerate an unpleasant medical examination such as bronchoscopy.
- To terminate anaesthesia induced and maintained by benzodiazepines.
- As a diagnostic help in cases of coma of unknown origin; if the patient does not wake up after being given 2 mg of flumazenil *i.v.*, benzodiazepine overdose can be ruled out. It is evident that flumazenil also works in the case of intoxication with zopiclone or zolpidem, since these two hypnotics that do not have the structure of a benzodiazepine nevertheless bind to the BZR.

3. The Aromatic Substitution

At the very beginning of this programme it became clear that Cl-substitution at C(8) is optimal only for the older benzodiazepines, like diazepam, but not for the series of the antagonists. Surprisingly, the fluoro-substituted flumazenil is more potent than the unsubstituted compound **14**. Therefore, we wondered whether this might in fact be the optimum position. In order to clarify this we synthesized all possible Cl-substituted isomers **24**–**27**.

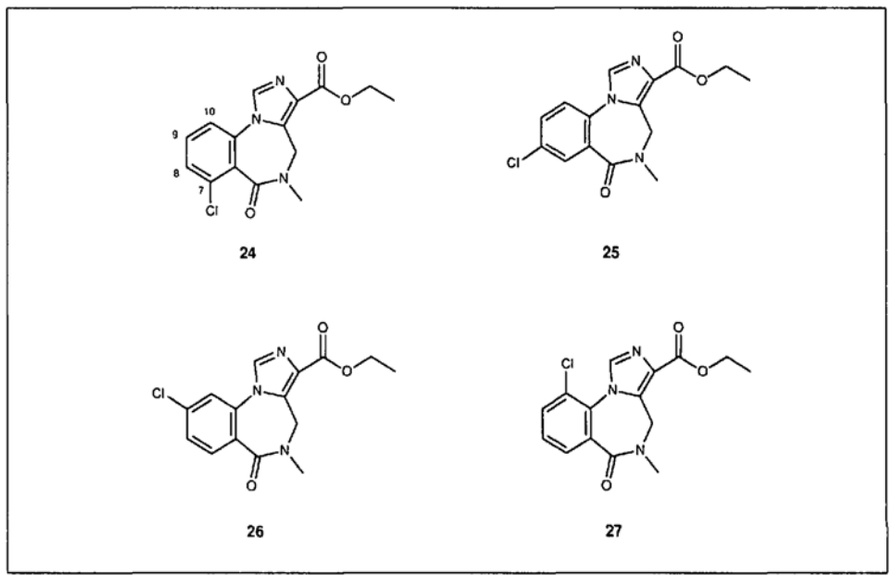
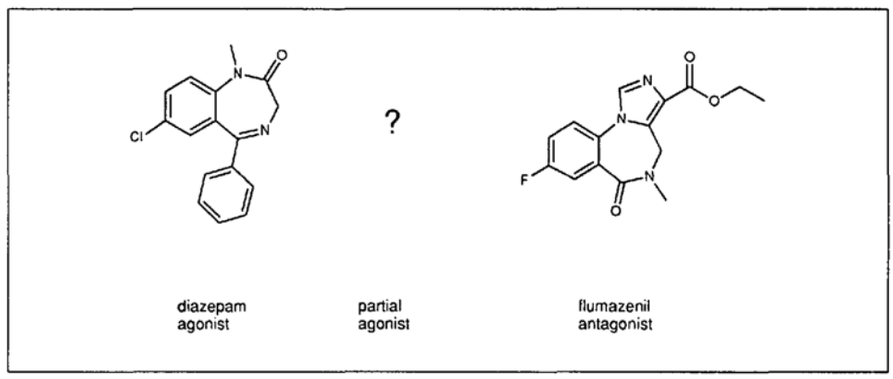


Table 2. *Influence of Substitution of the Phenyl Ring on the Pharmacological Activity*

Compound	Inhibition of ^3H-diazepam binding *in vitro*, IC_{50} in nM	Antagonism of diazepam *vs.* pentylenetetrazole, mice, *p.o.*, ED_{50} in mg/kg
24	2.7	0.4
25	6.8	32.5
26	> 1000	100 inact
27	> 1000	100 inact

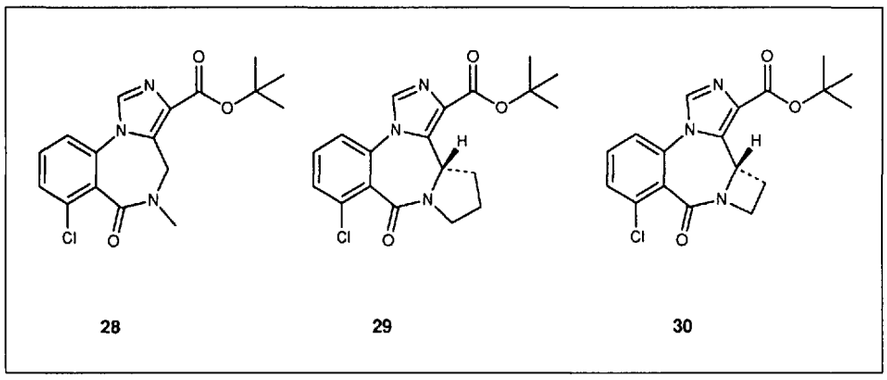


We found a major deviation from the situation with classical benzodiazepines in that the isomer substituted at position seven (**24**) was 40 times more potent in a test for antagonism than the one substituted in position eight (**25**). The isomers substituted at C(9) (**26**) or at C(10) (**27**) were inactive (*Table 2*). Consequently, we concentrated on the synthesis of compounds with chlorine in position seven.

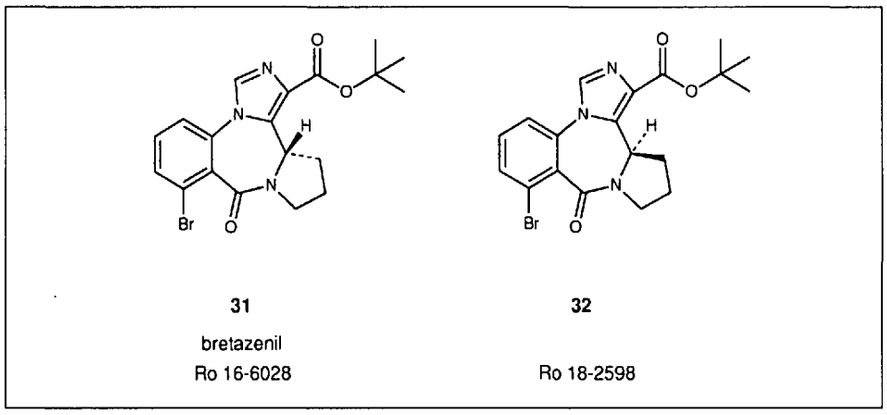
4. Serendipity Again. Partial Agonists

If there are agonists and antagonists for a particular receptor, there should also be ligands in the range between the two, or in pharmacological terms, with an intermediate intrinsic efficacy. On the one hand, we had agonists like diazepam (**2**) and on the other hand, antagonists like flumazenil (**20**). What would a partial agonist look like? Frankly we had no idea.

The methyl, ethyl, and isopropyl esters are all powerful antagonists. However, with the propyl ester the potency drops

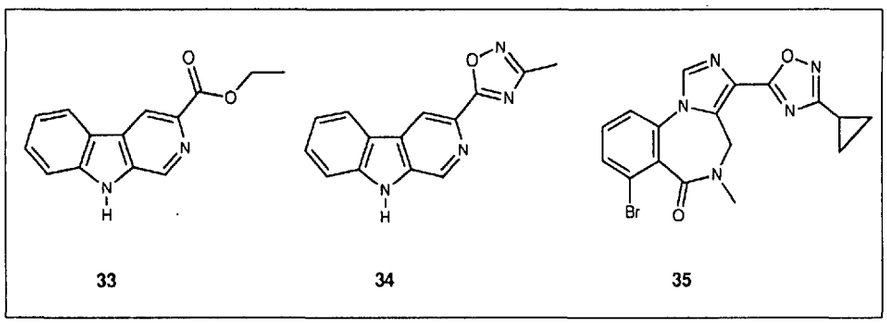


28 29 30

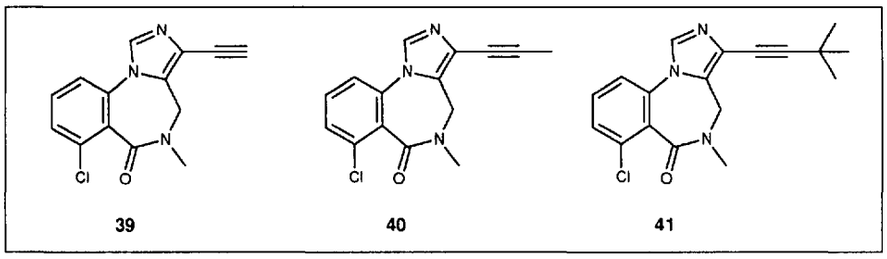


31
bretazenil
Ro 16-6028

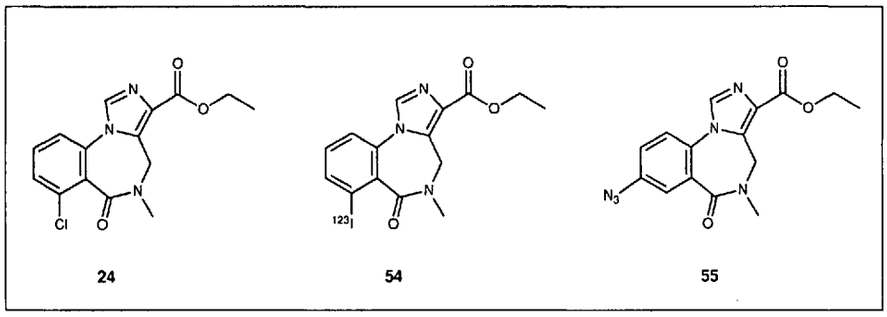
32
Ro 18-2598



33 34 35



39 40 41



24 54 55

tenfold. The hexyl ester still has an excellent affinity for the BZR *in vitro*, but is virtually inactive *in vivo*. The length of the ester is obviously restricted to C_2. If this is true, the *tert*-butyl esters (the dimethylethyl esters) should, by rights, also be potent antagonists. So we synthesized the *tert*-butyl ester **28** with optimum aromatic substitution and found that it is one of the most potent antagonists – at least ten times more potent than flumazenil (**20**). We immediately synthesized both tetracyclic analogues but the results were disappointing: both were completely inactive in the most important test for antagonism (*Table 3*).

However, in the receptor-binding test they were just as potent as the tricyclic compound **28**. Could they be agonists? Compound **29** was found to lack agonistic activity against pentylenetetrazole-induced seizures in mice. In spite of this, the compound was examined in the conflict test in rats: this time it proved to be considerably more potent than diazepam (**2**). The anti-pentylenetetrazole test was repeated in rats, and the compound was 75 times more potent than diazepam (**2**) in the same species. Apparently we were dealing with a difference between animal species. As it turned out, this class of compounds also works in humans.

Had we discovered nothing more than a new but more potent diazepam? No, it was the first partial agonist acting at the BZR. In some tests, *e.g.* in the horizontal wire test, **29** antagonized the effect of the full agonist diazepam (**2**) (*Fig. 1*).

In this test the animals are lifted by the tail and allowed to grasp a horizontally strung wire with their forepaws and are then released. Untreated control animals heave themselves onto the wire with at least one hindpaw. Diazepam (**2**) impairs this performance (right side). Flumazenil (**20**) given 15 min after diazepam (**2**) restores control behavior (left side) [10].

4.1. *Bretazenil* (31)

Bretazenil (**31**), the bromo analogue of **29** was selected for clinical development [11]. It produces anticonflict effects, indicative of anxiolytic activity in man, at much lower doses and over a much wider dose range than diazepam. It is also a very potent anticonvulsant. Apart from these effects, the following additional advantages were revealed by animal experiments:

- strongly reduced sedative activity
- virtually no disturbance of motor control
- no development of tolerance
- no development of physical dependence

– greatly reduced potentiation of the sedative effects of alcohol

For a thorough discussion of the concept of partial agonism see [12].

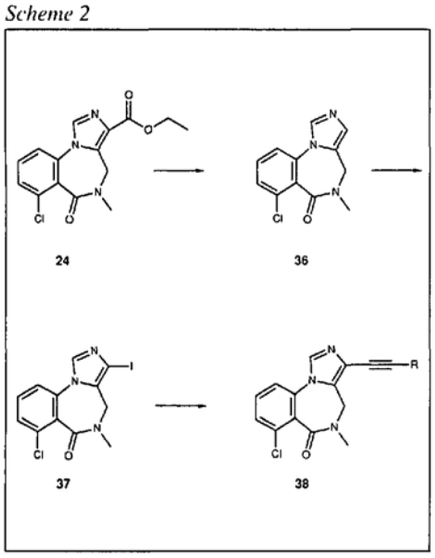
Bretazenil (**31**) is a chiral compound. Its configuration is (*S*). The (*R*)-enantiomer **32** has 10,000 times less affinity for the BZR and was not active in the pharmacological tests at the doses shown (*Table 4*). Incidentally, it costs less to synthesize the pure (*S*)-compound than the racemic compound, because natural (*S*)-proline is cheaper than racemic proline.

5. 1,2,4-Oxadiazoles

As esters are metabolically unstable, their duration of action is relatively short. Two hydrogen-bond acceptors appear to be optimal, since the ketones and ethers corresponding to the esters are considerably less potent. We were looking for an alternative. In the case of β-carbolines (**33**) it was possible to replace the ester by various heterocycles, *e.g.* by 1,2,4-oxadiazoles **34** [13]. In the case of the benzodiazepines, this concept also worked well. The pharmacological profile was shifted towards more agonism. Soon after our patent application was filed [14] an application by a competitor was published [15].

We found the cyclopropyl-substituted 1,2,4-oxadiazoles very interesting and, to our relief, they were not the object of a claim by our competitor. Their duration of action is longer than that of the corresponding methyl derivatives. The reason for this might be that the formation of radicals, intermediates in the pathway of cytochrome-P450-catalyzed metabolism, is much more difficult in the case of cyclopropane than an alkane like 2,2-dimethylpropane [16].

Scheme 2



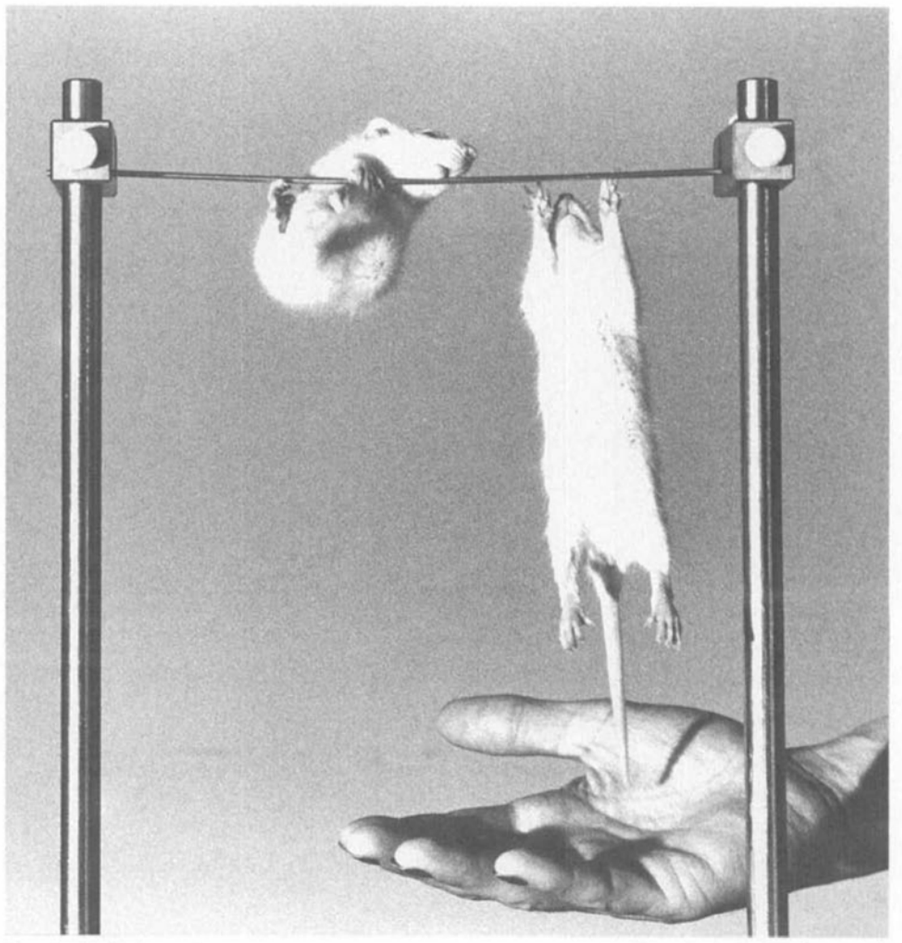


Fig. 1. *The horizontal wire test*

Table 3. *A Small Structural Difference with an Important Change in Pharmaceutical Effect*

Compound	**28**	**29**	**30**
Binding	3.0	2.5	5.3
Antagonism	0.2	100 inact	50 inact
Agonism	100 inact	0.29	0.35
HWT Antagonism	0.004	2.6	30 slact

Binding: Inhibition of ^3H-diazepam binding *in vitro*, rats, IC_{50}, nM.
Antagonism: Antagonism of diazepam protection *vs.* pentylenetetrazole, mice, ED_{50} in mg/kg, *p.o.*
Agonism: Prevention of pentylenetetrazole-induced convulsions, rats, ED_{50} in mg/kg, *p.o.*
HWT: Antagonism of diazepam in the horizontal wire test, rats, ED_{50} in mg/kg, *p.o.*

Table 4. *Comparison of the Two Enantiomers* **31** *and* **32**

Compound	**31**	**32**
Binding	2.2	25000
Agonism	0.07	100 inact
HWR Antagonism	0.3	50 inact
Conflict Test	0.02	80 inact

Binding: Inhibition of ^3H-Diazepam binding *in vitro*, rats, IC_{50}, nM, *p.o.*
Agonism: Prevention of pentylenetetrazole-induced convulsions, rats, ED_{50} in mg/kg, *p.o.*
HWT: Antagonism of diazepam in the horizontal wire test, rats, ED_{50} in mg/kg, *p.o.*
Conflict test: Fixed ratio 1 conflict, rats, FSD in mg/kg, *p.o.*

Scheme 3

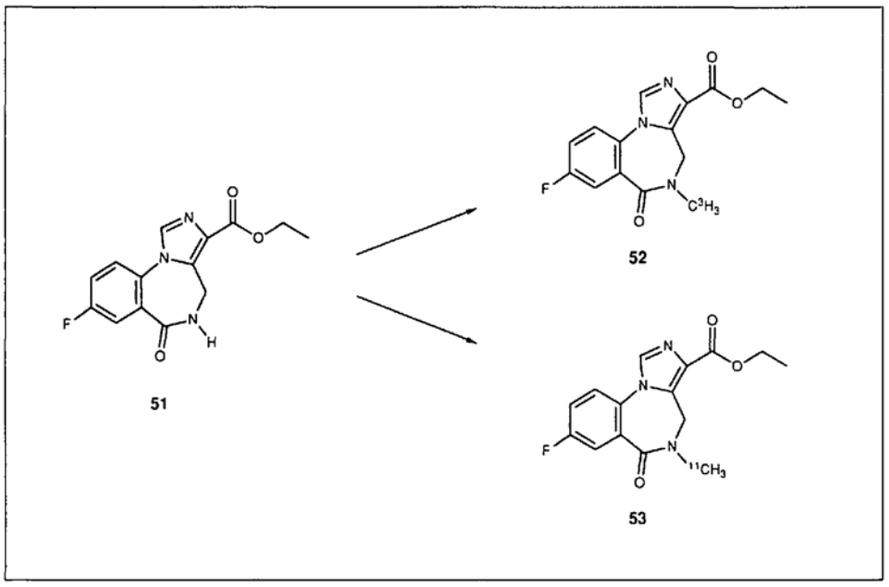


Table 5. *Pharmacological Results of the Alkyne Derivatives* **39–41**

Compound	Inhibition of ^3H-flumazenil binding *in vitro*, IC_{50}, nM	Antagonism of diazepam protection *vs.* pentylenetetrazole, mice, *p.o.*, ED_{50} in mg/kg	Prevention of audiogenic tonic convulsions in DBA/2J mice, *p.o.*, ED_{50} in mg/kg
39	1.6	0.24	100 inact
40	1.1	0.13	49
41	2.1	50 inact	0.89

6. The Rational Approach Finally? No, But Still More Serendipity. Alkynes

We were convinced that high affinity for the BZR can be achieved best if the substituent on the imidazolo ring has two hydrogen-bridge acceptors. Esters and 1,2,4-oxadiazoles are typical examples. Replacement of these cornerstones by alkynes is, therefore, not logical at all. Nevertheles we went ahead. Was it the pure joy of chemistry in action?

The ester **24** is hydrolyzed to the acid, which is decarboxylated to **36**. Iodine is introduced regiospecifically at C(3) with iodine in DMF, $CaCO_3$ as base (→**37**), which reacts smoothly with a variety of alkynes in DMF with triethylamine as base and bis(triphenylphosphine)palladium(II) dichloride and copper(I) iodide as catalysts to give compounds of type **38** in yields ranging from 60 to 90% [17].

The pharmacological results were exiting (*Table 5*). Not only did this new series of compounds have a high affinity for the BZR, they also displayed a wide variety of pharmacological profiles. **39** is a partial inverse agonist, **40** an antagonist with very low intrinsic activity and **41** a partial agonist.

7. Structure-Activity Relationship

The structure-activity relationship in this series of benzodiazepines is very in-

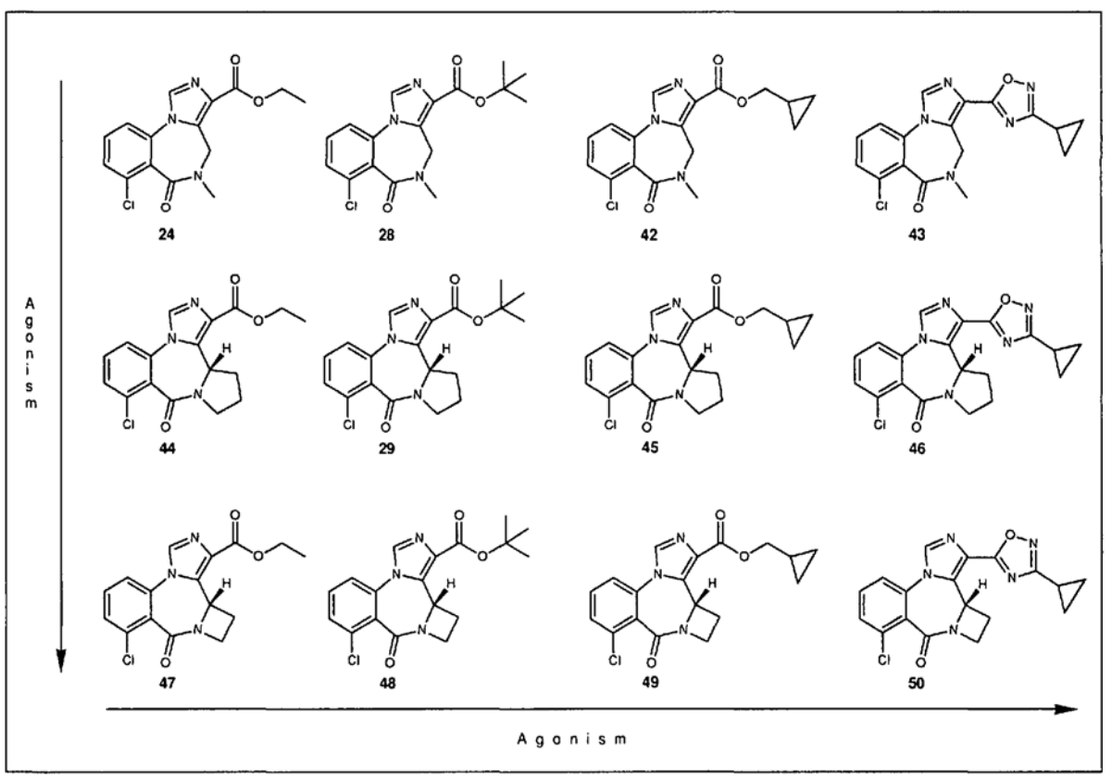


Fig. 2. *Structure-activity relationship*

teresting. It is fascinating how the slightest modification affects the pharmacological profile.

Fig. 2 shows twelve compounds: the ethyl, *tert*-butyl and cyclopropylmethyl esters as well as the cyclopropyl-oxadiazoles. In the top row are the tricyclic compounds, followed by the tetracyclic compounds with the five-membered ring and those with the four-membered ring. The agonistic component increases progressively from top to bottom and from left to right. **24** is not a pure antagonist, as it also has proconvulsant properties. Its neighbors, the tricyclic *tert*-butyl ester and the tetracyclic ethyl ester are pure antagonists. By combining the two structural elements we obtain the partial agonist **29**, the chloro analogue of bretazenil (**31**). One step down and one to the right we have **49** which was, under the code number *Ro 17-1812*, for a short time in clinical trial [18]. It is definitely more agonistic than **29**. Compound **50** at bottom right is a full agonist. It is unable to antagonize diazepam in the horizontal wire test, as it produces an agonistic effect itself in this test.

8. Spin-offs

Flumazenil (**20**) is widely used as a tool in CNS pharmacology. If an effect cannot be antagonized with flumazenil it is not mediated by the BZR. ^{3}H-Flumazenil is a very good ligand for *in vitro* and *in vivo* studies [19] because the nonspecific binding is low.

^{11}C-Flumazenil is an excellent ligand for positron emission tomography (PET), which makes it possible to show the presence and the distribution of the BZR in man [20][21].

Both labelled compounds are synthesized from **51** either by alkylation with $C^{3}H_{3}I$ or with $^{11}CH_{3}I$ (*Scheme 3*).

^{123}I-Iomazenil (**54**) is used as ligand for the BZR in single photon emission computed tomography (SPECT). **54** is also used as a brain imaging agent in humans [22–24]. It is on the market in Europe.

Sarmazenil (**24**) is licensed out as a benzodiazepine antagonist for use in veterinary medicine after anaesthesia induced with a benzodiazepine [25].

The azido derivative **55** was synthesized as a photoaffinity label for the BZR. Under UV-irradiation it binds covalently to the receptor [26]. The ^{3}H-labelled derivative is commercially available as ^{3}H *Ro 15-4513*. Yet, *Ro 15-4513* is better known for other reasons: When its pharmacological profile was elaborated it was also tested for possible interaction with the effects of other CNS depressants, like barbiturates and ethanol, in the afore-mentioned horizontal wire test.

In contrast to flumazenil (**20**) *Ro 15-4513* was active against some effects of some doses of phenobarbitone and ethanol. These results were presented as posters at a meeting of the British Pharmacological Society in Southampton [27]. Approximately one year later the lay press made up a big story out of this compound as a possible alcohol antagonist or sobering-up pill. The development of such a pill was never considered by us for many reasons, the clinical most important one that the toxicity of ethanol is not reduced by *Ro 15-4513* [28]. Nevertheless, it remains an interesting tool in pharmacology [29][30].

Conclusion

The benzodiazepines have been around for more than 30 years, however, their medicinal chemistry and pharmacology have never been more exciting than today. To end with the author's credo: Creativity and fun are inseparable.

The development of the antagonists and partial agonists was the result of exciting teamwork. I thank my colleagues for their invaluable contributions. Special thanks go to *Marc Meier* and *Beatrix Stauffer* who, in my laboratory, synthesized all new benzodiazepines mentioned in this paper and well over 1000 additional compounds in connection with this project.

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