Full Paper

Synthesis of Substituted 3-Anilino-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepine-2-ones and their Evaluation as Cholecystokinin-Ligands

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3-Amino-1,4-benzodiazepines as well as chemically related diverse amines were prepared from oxazepam and subsequently screened on the cholecystokinin receptor in a radiolabel binding assay. Oxazepam **2** was activated *via* its 3-chloro-1,4-benzodiazepine intermediate **3** and was reacted with a large series of aliphatic and aromatic amines. The substituted 3-anilino-1,4-benzodiazepine structure was identified as lead structure in a diverse series of 3-amino-1,4-benzodiazepines **4**-**38** and the full SAR (structure-activity relationship) optimisation provided 3-anilinobenzodiazepines **16**–**38** with CCK_1 receptor selectivity to CCK_2. The compounds **18**, **24**, **28** and **33** have shown affinities at the CCK_1 receptor of 11, 10, 11 and 9 nM, respectively. These equipotent CCK_1 ligands were fully evaluated in behaviour pharmacological essays. An antidepressant effect was identified in the tail suspension- and the Porsolt swimming-test. The ED_{50} values for **24** and **28** were determined in these assays as 0.46 and 0.49 mg/kg. The mixed antagonist **37** showed in addition to the antidepressant effects anxiolytic properties.

Keywords: 1,4-Benzodiazepines / CCK receptor / Cholecystokinin / Antidepressant

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Introduction

Cholecystokinine (CCK), which act as a neuromodulator/gut hormone and CCK-ligands, agonists as well as antagonists [1], have been extensively investigated as potential drug targets [2]. Antagonists were studied as growth inhibitors in certain forms of cancer [3], as anxiolytics [4], in the treatment of schizophrenia [5], satiety [6] and as antipanic agents [7]. An agonist, the shortened CCK tetrapeptide, was found to induce panic in patients [8]. A phase II clinical trial of devazepide, a potent and CCK_1 selective antagonist [9], has been recently completed showing a significant enhancement of the effect of morphine in the treatment of chronic and severe pain [10]. In order to fully understand, why it was focussed again on the class of 1,4-benzodiazepines, a brief overview on different classes of cholecystokinin ligands is presented. Therefore, an early CCK antagonist, lorglumide, an example of a peptidal antagonist, CI 988, the CCK_1 selective standard devazepide and YM 022, an optimised Merck ligand, are outlined in Figure 1.

Lorglumide is a CCK_1 selective antagonist with a low potency and presents chemically an amide derivative of glutamic acid [11, 12]. A research group at Parke-Davis [13] examined the activity of CCK-30-33 fragments in binding experiments on CCK_2/gastrin receptors. This led to the development of CI 988, which exhibited a 1600-fold selectivity for CCK_2 over CCK_1 receptors with a subnanomolar affinity (IC_{50} = 0.3 nM). However, due to the high molecular weight and to the dipeptoid structure these derivatives have a low bioavailability and are not suitable for oral therapy [14].

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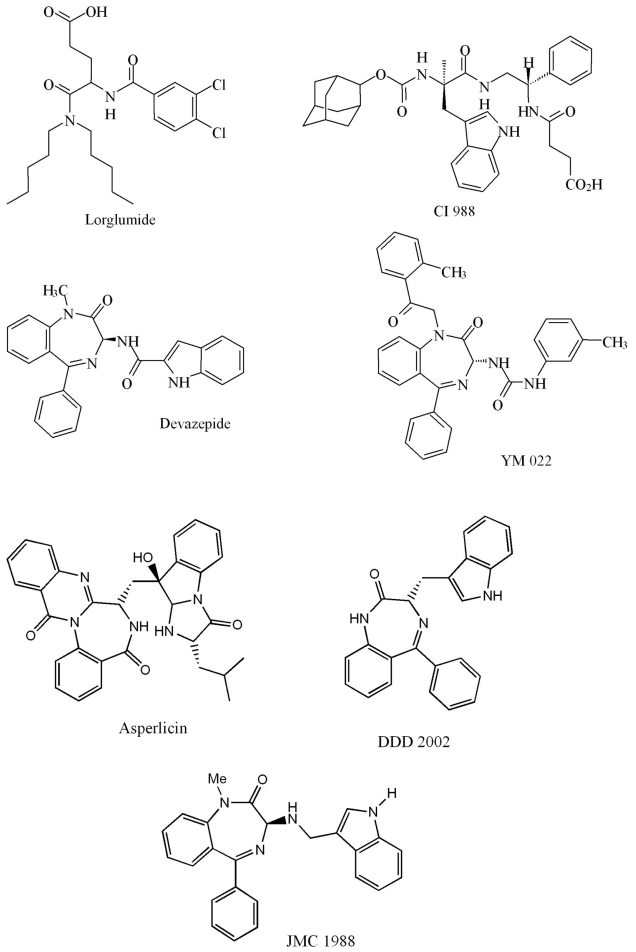


Figure 1. Overview on different classes of cholecystokinin ligands.

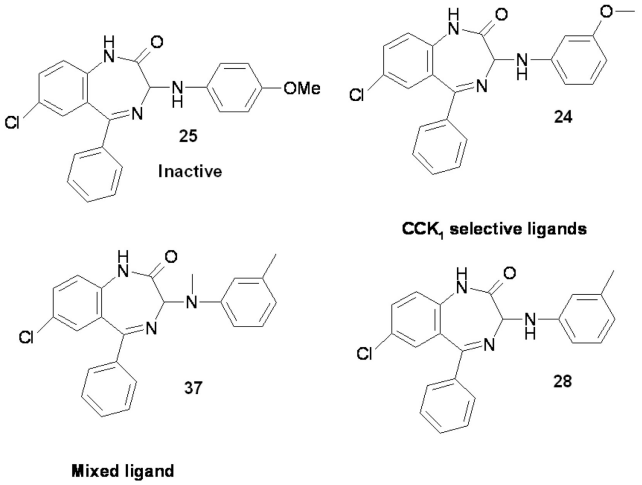


Figure 2. Overview of selected CCK-antagonists.

L-364, 718 or MK-329 or devazepide revealed nanomolar affinity for peripheral receptors, longer lasting efficacy *in vitro* and *in vivo*, whilst orally bioavailable. devazepide [15, 16] possessed potent CCK_1 blocking activity in different tissues. Pancreatic amylase secretion is antagonised with a potency of 2 000 000 times more than lorglumide. Devazepide has been claimed to be a selective antagonist of the effects of CCK-8 on food intake [17] and it was a key tool in the autoradiographical demonstration of the presence of CCK_1 receptors in the various regions of the brain [18].

A novel potent series of 1-arylmethyl analogues of L-365, 260 [19] was prepared in complex synthetical steps, but the water solubility still remained a problem [20, 21].

Our early work on 1,4-benzodiazepines, based on asperlicin, provided asperlicin- analogues [22] and other libraries of *N*-alkylated 3-propyl-1,4-benzodiazepines. With two alkyl chains, a CCK_2 selectivity was achieved, combined with a very high lipophilicity [23]. Various work was reported using the 3-amino-1,4-benzodiazepine template [24]. The only example of CCK ligands in the class of substituted arylated or alkylated amines, attached to the 3-position was a 3-amino-2-indolylmethyl-1,4-benzodiazepine (CCK_1:IC_{50} = 87 nM) of in total two examples of 3-amino-benzodiazepines with a high binding affinity [25]. This exciting structure served as our lead structure of CCK_1 selective ligands, when their relevance was understood as agents in the treatment of depression [26].

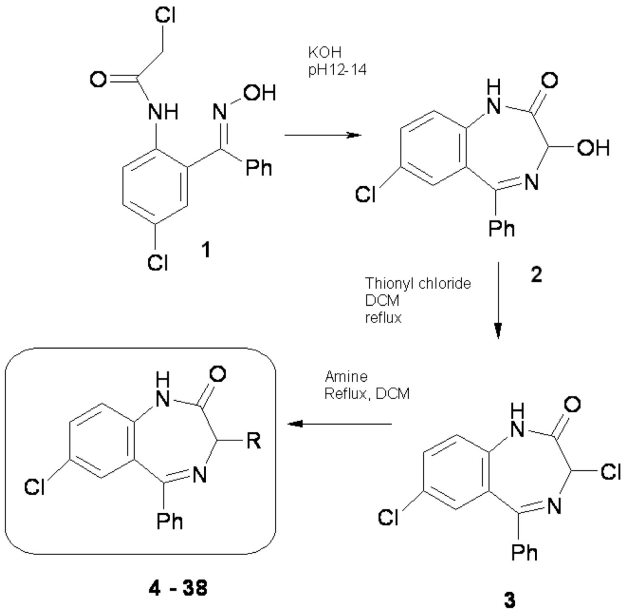
Here, we report the synthesis, binding affinity and *in vivo* studies in mice on novel, CCK_1 selective antagonists with a much improved water solubility compared to the ureido-1,4-benzodiazepines.

Chemistry

Various approaches towards oxazepam were available, such as the oxidation of the 3-position of diazepam [27]. Oxazepam was claimed to be synthesized *via* the known Polonowski rearrangement of the *N*-oxide on an industrial scale [28]. The chloroacetamide amide **1** was converted into a quinazoline structure [29] in presence of a Lewis acid, such as $ZnCl_2$.

The formation of the desired benzodiazepine structure **2** took place from acetamide **1** under controlled alkaline conditions enabling us to synthesise gramme quantities of this key intermediate. The chloro-acetamide **1** was cyclised into the desired oxazepam salt at ambient temperature with an alcoholic solution of KOH at pH 11–14, which was subsequently titrated with 3 M HCl to pH 1.8 to give the free oxazepam **2**.

An activation of the 3-hydroxy group with DEAD *via* phosphono-oxy derivatives was described [30], but the most reliable route towards the 3-amino-1,4-benzodiaze-



Scheme 1. Synthesis route of compounds **4–38**.

pines was found *via* the 3-chloro-1,4-benzodiazepine **3**. Oxazepam **2** was reacted with an excess of thionyl chloride to give a yellow 3-chloro-1,4-benzodiazepine **3**, which was reacted *in situ* with the corresponding amine into the 3-aminobenzodiazepines **4–38** in presence of a catalytic amount of TEA. Amines **4–16** were purified by chromatography, while the anilines **17–38** precipitated out after the addition of *n*-hexane to the reaction mixture.

Results and discussion

SAR studies

N-alkylated derivatives **4–6** showed a binding affinity above 20 mM; piperazines such as benzodiazepine **7** and the substituted aminopiperidine **8**, as an example, showed a very weak binding affinity towards the CCK receptor. Removal of substituents on the aromatic system (entry **16**) and the replacement of the aromatic phenyl group by a cyclohexyl system (entry **13**) resulted in a reduced binding affinity. Ring closure of the aniline structure into the quinoline derivative **9** was found less active. A CH_2-spacer between the benzodiazepine template and the aniline structure, as for benzyl derivative **10** displayed a decreased binding affinity and the phenylethyl-analogue **11** showed a loss of receptor binding. The unsubstituted 3-anilino 1,4-benzodiazepine **16** itself displayed a low binding affinity, but the aniline structure was clearly obtained as a privileged structure from this first series (entry **4–16**). An overview about the yields and the receptor binding affinity towards the CCK receptors are given in Table 1.

Following these initial findings, a large series of 3-anilino-1,4-benzodiazepines **17–38** were prepared in order to fully explore the structure activity relationships (SAR).

It was found that a *meta*-phenyl substituent had increased binding affinity: the *m*-nitro, *m*-methoxy and the *m*-methyl anilino derivatives **18**, **24** and **28** showed an equipotent affinity towards the CCK_1 receptor of 10 and 11 nM.

Interestingly, the *m*-choroderivative **21** displayed only a tenth of this binding affinity. The dimethylanilino-analogue **33** was found of the same affinity, if both substituents were attached to two *meta* positions. *N*-Methylation gave **37**, a highly potent mixed CCK_1/CCK_2 antagonist. The introduction of an *N*-ethyl group resulted in a decreased binding affinity (entry **38**).

In vivo pharmacology

The *p*-methoxy derivative **25** was found inactive in all *in vivo* assays, as expected, in correlation with no receptor binding activity. The CCK_1 antagonists **24**, **28**, **31** and **33** have shown a MED (minimum effective dose) of 0.1 mg/kg for the tail suspension test and the forced swim test in mice. The mixed cholecystokinin antagonist **37** has shown the same antidepressant bioactivity and in addition, an anxiolytic effect was determined in the elevated x-maze and the light/dark box test for the same dose. No analgesic effect and no impairment of coordination were observed in a group of 5 mice up to a dose of 20 mg/kg for the series outlined in Table 2.

After the first evaluation, the determination of the MED, the two CCK_1 selective ligands **24** and **28** and mixed CCK-ligand **37** were chosen for a full data collection. The nitro derivative **18** was not tested further due to a possible metabolism to a potentially toxic nitroso compound.

The *m*-toluene derivative **28** and the *m*-methoxy derivative **24** were evaluated at the same range using a group of 10 mice for each dose. An ED_{50} of 0.46, 0.46 mg/kg was determined for the tail suspension test and an ED_{50} of 0.49, 0.49 mg/kg was found for the Porsolt swimming test.

The mixed CCK antagonist **37** showed in addition to the antidepressant effects an ED_{50} of 0.46 and 0.42 mg/kg for the elevated x-maze and for the light/dark box test, respectively. Note: All three ligands antagonised CCK-8s and CCK-4 induced contractions of the gall bladder and the guinea pig ileum [31].

Conclusions

Chemical diversity in the relevant 3-position of the non-toxic 1,4-benzodiazepine scaffold was used to replace the

Table 1. Binding affinity of 3-amino-1,4-benzodiazepines for the CCK receptor.

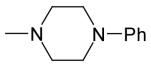
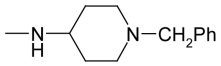
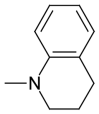
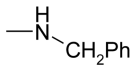
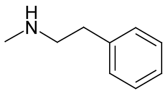
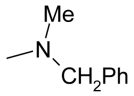
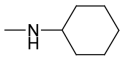
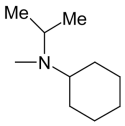
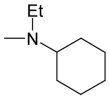
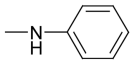
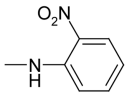
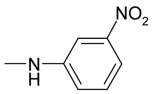
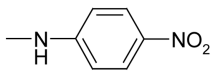
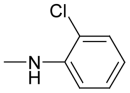
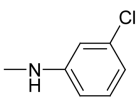
Entry	Structure: R	MS (M+1) [m/z]	Yield [%]	IC_{50} [μM] CCK_2	IC_{50} [μM] CCK_1
4	ethyl	315	38	>20	>20
5	*n*-propyl	329	47	>20	>20
6	*n*-butyl	343	61	>20	>20
7		431	58	15	>20
8		459	35	15	>20
9		402	66	7.2	>20
10		376	62	6.0	>20
11		390	60	>20	>20
12		390	36	2.9	>20
13		368	33	10.1	>20
14		410	31	>15	>20
15		396	30	>15	>20
16		362	80	4.1	>20
17		407	49	7.5	>20
18		407	68	6 ± 1	0.011 ± 0.002
19		407	74	8.5	>20
20		396	41	9.3	>20
21		396	53	6.2	0.27

Table 1. Continued.

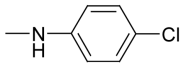
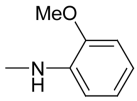
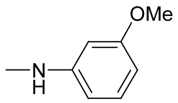
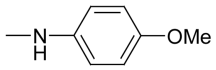
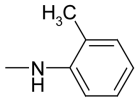
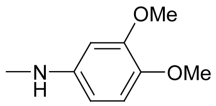
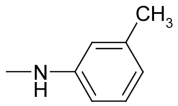
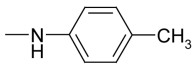
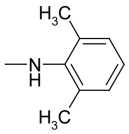
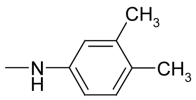
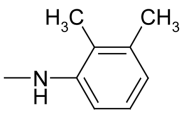
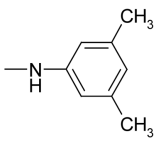
Entry	Structure: R	MS (M+1) [m/z]	Yield [%]	IC_{50} [μM] CCK_2	IC_{50} [μM] CCK_1
22		396	67	2.9	20
23		392	40	1.5	>20
24		392	45	4.5 ± 0.4	0.010 ± 0.002
25		392	62	11 ± 2	20 ± 2
26		376	48	7.1	10
27		422	50	1.2	>20
28		376	85	3.8 ± 0.5	0.011 ± 0.002
29		376	92	4.6	0.29
30		390	22	>10	10
31		390	48	0.92 ± 0.05	0.015 ± 0.002
32		390	46	6 ± 1	0.018 ± 0.002
33		390	72	2.9 ± 0.5	0.009 ± 0.002

Table 1. Continued.

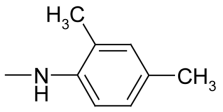
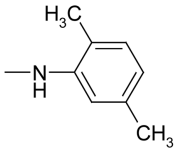
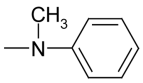
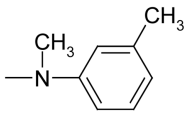
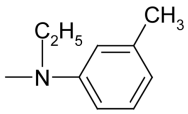
Entry	Structure: R	MS (M+1) [m/z]	Yield [%]	IC_{50} [μM] CCK_2	IC_{50} [μM] CCK_1
34		390	67	6.1 ± 0.5	0.39 ± 0.05
35		390	56	6.0 ± 0.5	1.0 ± 0.3
36		376	39	0.15 ± 0.05	0.014 ± 0.002
37		390	39	0.072 ± 0.008	0.008 ± 0.002
38		404	48	0.65 ± 0.04	0.33 ± 0.04

Table 2. *In vivo* evaluation of selected 3-anilino-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepine-2-ones.

Compound	Receptor binding IC_{50} [nM]		Elevated plus-maze	Light/dark box	Tail suspension test	Forced swim test	Thermal tail flick test	Hot plate test	Rota-rod test	Wire mesh grasping test
	CCK_1	CCK_2								
24	10 ± 2	4 500 ± 400	NS	NS	0.1	0.1	NS	NS	NS	NS
25	11 000 ± 2000	20 000 ± 2000	NS	NS	NS	NS	NS	NS	NS	NS
28	11 ± 2	3 800 ± 500	NS	NS	0.1	0.1	NS	NS	NS	NS
31	15 ± 2	920 ± 50	NS	NS	0.1	0.1	NS	NS	NS	NS
33	9 ± 2	2 900 ± 500	NS	NS	0.1	0.1	NS	NS	NS	NS
37	8 ± 2	72 ± 8	0.1	0.1	0.1	0.1	NS	NS	NS	NS

NS = no significance could be observed at 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 mg/kg; MED [mg/kg]: Minimum effective dose.

Table 3. *In vivo* studies of CCK antagonist **24**, **28** and **37** in mice.

	24 ED_{50} [mg/kg]	**28** ED_{50} [mg/kg]	**37** ED_{50} [mg/kg]
Elevated plus maze	–	–	0.46 ± 0.15
Light/dark box	–	–	0.42 ± 0.20
Tail suspension test	0.46 ± 0.12	0.46 ± 0.11	0.50 ± 0.20
Forced swim test	0.49 ± 0.13	0.49 ± 0.11	0.48 ± 0.21

known urea linkage by a novel 3-anilino-lead structure. The CCK_1 selective antagonists displayed antidepressant properties in two standard assays carried out in mice. The mixed cholecystokinin antagonist **37** has shown antidepressant, as well as anxiolytic properties. Our compounds occured a better water solubility than the most recent generation of ureas containing a cationic solubilising group [32]. The resolution of salts of maleic acid is currently under investigation and will provide enantiomerically pure pharmaceuticals.

We acknowledge Dr. David Poyner for providing pancreatic and brain cortex membranes and Mr. Simon Dunn for in vitro testing of ligand 28, 24 and 37. This work was partially supported by the EPSRC and Panos Therapeutics Ltd.

Experimental

Chemistry

General methods

Chemicals were purchased from Aldrich UK and Lancaster Ltd (Aldrich, Gillingham, UK; Lancaster Synthesis, Morecambe, UK). Mass spectra were obtained by Atmospheric Pressure Chemical Ionisation (APCI), negative or positive mode, using a Hewlett-Packard 5989b quadrupole instrument (Hewlett-Packard, Palo Alto, CA , USA). Samples were dissolved in HPLC grade methanol, toluene or acetonitrile. Proton and Carbon NMR spectra were obtained on a Bruker AC 250 instrument (Bruker, Coventry, UK) calibrated with the solvent reference peak or TMS.

IR spectra were plotted from KBr discs on a Mattson 300 FTIR Spectrophotometer (Mattson Instruments, Harston, UK). Melting points were recorded from a Stuart Scientific Melting Point (SMP1; Fisher Scientific, Loughborough, UK) and are uncorrected. Analytical Thin Layer Chromatography was obtained using aluminium sheets, silica gel 60, F_{254} and visualised using ultraviolet light. Preparative chromatography was performed on 250 μm, 20 × 20 cm silica gel TLC plates, obtained from Aldrich. Jencons sonomatic sonicator (SO175; Fisher Scientific) was used to prepare samples for screening. All compounds were dissolved in DMSO for screening. Small scale solution syntheses were carried out on a carousel reaction stations (RR 98030; Fisher Scientific), fitted with a 12 place carousel reaction station.

2-Chloro-N-{4-chloro-2-[(hydroxyimino)(phenyl)methyl]phenyl} acetamide ***1***

A solution of 2-amino-(5-chlorophenyl)(phenyl)methanoneoxime (142 mmol, 35.18 g) in ether (1000 mL) and water (300 mL) was stirred in an ice bath at 0–5°C. Chloroacetyl chloride (160 mmol, 12.8 mL) was added dropwise, over 30 min, whilst maintaining a slightly basic solution with the addition of 15% aqueous sodium hydroxide. After the addition of chloroacetyl chloride the reaction was stirred for an additional 3 h at room temperature. The organic phase was washed with water, dried over magnesium sulphate and concentrated to dryness to yield a white powder.

Yield: 94%; IR (KBr) cm^{-1}: 3390, 3300, 3015, 2900, 1660, 820; MF $C_{15}H_{12}N_2O_2Cl_2$; MW 323.2; MS (APCI(+)): 323, 325 (M+1), 305, 307 (-$H_2$0) m/z; ^{1}H-NMR (DMSO-d_6) 300K δ: 11.92 (s, N– OH), 9.25 (s, NH), 7.39 (s, phenyl-H), 7.20 (s, C3-H), 7.53 (d, C6-H, *J* = 8.7 Hz), 7.83 (d, C5-H, *J* = 8.8 Hz), 4.06 (s, $-CH_2-$) ppm; ^{13}C-NMR (DMSO-d_6) 300K δ 157.2 (C=N–OH), 137.1 (C–NH–), 43.1 ($-CH_2-$), 136.9, 136.0, 133.9, 133.1, 132.8, 132.2, 130.8, 130.1,127.9, 125.8, 124.9 (Ar-C) ppm.

7-Chloro-3-hydroxy-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***2***

A solution of 2-chloro-*N*-{4-chloro-2-[(hydroxyimino)(phenyl)-methyl]phenyl} acetamide (134 mmol, 43.28 g) in ethanol (900 mL) and sodium hydroxide (2 M, 280 mL) were stirred at room temperature over night. The precipitate that formed was separated by filtration and dissolved in a minium amount of ethanol/water 60 : 40 mix, (undissolved oxazepam salt was collected and dried). The mixture was acidified to pH 1.0–2.0 by the addition of concentrated hydrochloric acid. The filtrate was cooled in a ice bath to 0–10°C over night. The precipitate was filtered and dried to give the crude product (light brown solid).

Yield: 59.8% (crystallisation with ethanol or 1,4 dioxane); IR (KBr) cm^{-1}: 3385, 3025, 3320, 3010, 1705, 1590, 730; MF $C_{15}H_{11}N_2O_2Cl$; MW 286.7; MS (APCI(+)): 287, 289 (M+1), 269, 271 (–H_2O) m/z; ^{1}H-NMR (DMSO-d_6) 300K δ: 10.81 (s, N–H), 7.48 (s, phenyl-H), 7.23 (s), 7.25 (d, Ar-H, *J* = 8.8 Hz), 7.64 (d, Ar-H, *J* = 8.7 Hz), 6.33 (d, OH, *J* = 8.7 Hz), 4.78 (d, C3-H, *J* = 8.7 Hz) ppm; ^{13}C-NMR (DMSO-d_6) 300K δ: 170.3 (C=N), 165.9 (C=O), 123.7, 127.1, 128.3, 128.5 (2 × C), 128.9, 129.7, 129.8 (2 × C), 131.0, 132.3, 138.5 (Ar-C), 83.3 (CH–OH) ppm.

3,7-Dichloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***3***

Oxazepam (0.5 g, 1.75 mmol) was treated with thionyl chloride (4 eq, 0.4 mL) and heated to 60°C for 1.5 h. The resulting intermediate, a yellow solid, was washed with dry diethyl ether (twice), to remove any excess of thionyl chloride.

Yield: 93.4%. MF: $C_{15}H_{10}N_2O_2Cl_2$. MW: 305.2. IR (KBr-disc) ν max: 3418, 3220, 3060, 2919, 1704, 1606, 1476, 1322, 1226, 902, 823, 693 cm^{-1}. MS (APCI(+)): 305, 306, 307 (M+1), 269, 270, 271 m/z. ^{1}H-NMR ($CDCl_3$) 300K δ: 9.89 (s, NH), 7.26–7.80 (m, Ar-H, 8H), 5.64 (s, C3-H) ppm.

General method for the preparation of 3-amino-substituted 1,4-benzodiazepin-2-ones ***4–38***

Oxazepam (0.1 g, 3.5 mmol) was treated with thionyl chloride (4 eq, 0.1 mL) and heated to 60°C for 1.5 hours. The resulting intermediate, a yellow solid, was washed with dry diethyl ether (twice) to remove any excess thionyl choride. The appropriate amine (2.5 eq, 1.1 mmol), with TEA (drops) were refluxed for two hours in DCM (15 mL). The organic phase was washed with hydrochloric acid and dried over sodium sulphate. Excess hexane was added and the mixture was allowed to stand overnight. The precipitate was filtered, washed with hexane and dried.

3-(Ethylamino)-7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***4***

Yield: 38%, mp.: 126–128°C, R_f (ether)= 0.40. MF: $C_{17}H_{16}ClN_3O$. MW: 313.8. IR (KBr-disc) ν max: 3430, 3121, 2977, 2855, 1654, 1607, 1478, 1320, 693 cm^{-1}. MS (APCI(+)): 314, 315 (M+1), 296, 297 (M+), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 1.16–1.24 (t, 3H, J = 7.1 Hz), 2.70-3.0 (m, $-CH_2-$), 4.34 (s, C3-H), 7.24–7.61 (m, Ar-8H), 11.19 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 14.7 (CH_3), 42.4 ($-CH_2-$), 71.3 (C3), 123.4, 125.8 (2 × C), 128.3, 129.7 (2 × C), 130.6, 131.1, 136.3, 137.7, 166.5 (C=O), 169.0 (C=N) ppm.

3-(Propylamino)-7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***5***

Yield: 47%, mp.: 128–130°C, R_f (ether)= 0.44. MF: $C_{18}H_{18}ClN_3O$. MW: 327.8. IR (KBr-disc) ν max: 3433, 3108, 2980, 2851, 1764, 1471 1315, 699 cm^{-1}. MS (APCI(+)): 326, 327 (M+1), 308, 309 (M+), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 1.11–1.20 (t, 3H, *J* = 7.0 Hz), 2.51–2.72 (m, 4H, $-CH_2-$), 4.45 (s, C3-H), 7.25–7.83 (m, Ar-8H), 11.12 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 12.9 (CH_3), 34.0 & 42.4 ($-CH_2-$), 71.1 (C3), 123.4, 125.1 (2 × C), 128.5, 129.9 (2 × C), 130.0, 131.3, 136.4, 137.7 (Ar-C), 166.2 (C=O), 169.5 (C=N) ppm.

3-(Butylamino)-7-chloro-5-phenyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one ***6***

Yield: 61%, mp.: 135–137°C, R_f (ethylacetate) = 0.54. MW: 341.8. IR (KBr-disc) ν max: 3425, 3100, 2930, 2850, 1700, 1640, 1615, 1480, 1330, 1080 cm^{-1}. MS (APCI(+)): 342, 344 (M+1), 324, 326 (–H_2O), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.57 (s, NH), 9.69 (s, NH), 7.77 (dd, Ar-H, *J* = 8.7 Hz), 7.60–7.49 (m, phenyl-5H), 7.42 (d, Ar-H, *J* = 8.8 Hz), 7.30 (s, Ar-H), 5.12 (s, C3-H), 3.21 (m, –CH–), 3.02 (m, –CH–), 1.73 (m, $-CH_2-$), 1.39 (m, $-CH_2-$), 0.92 (t, CH_3, *J* = 7.2, 7.4 Hz) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 13.7 (CH_3), 21.9, 30.2, 45.2 (CH_2), 70.5 (C3), 121.9, 125.1 (2 × C), 127.6, 128.2, 129.9 (2 × C), 130.8, 131.3, 132.0, 136.8, 138.2 (Ar-C), 167.5 (C=O), 168.1 (C=N) ppm.

3-(1-Phenylpiperazine)-7-chloro-5-phenyl-1,3-dihydro-2-H-1,4-benzodiazepine-2-one ***7***

Yield: 58%, mp.: 168–170°C, R_f (ethylacetate) = 0.37. MW: 430.94. IR (KBr-disc) ν max: 3434, 3049, 2921, 2417, 1704, 1596, 1482, 1324, 1091, 685 cm^{-1}. MS (APCI(+)): 431, 433 (M+1), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.79 (s, NH), 7.76–7.82 (dd, Ar-H, *J* = 9.8 Hz), 7.49–7.65 (m, phenyl-6H), 7.34 (s, Ar-H), 7.26–7.32 (d, Ar-2H, *J* = 8.3 Hz), 7.02–7.05 (d, Ar-2H, *J* = 8.1 Hz), 6.85–6.91 (t, Ar-H, *J* = 7.3, 7.2 Hz), 5.30 (s, C_3-H), 3.40–3.53 (m, $-CH_2-$ 8H) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 58.5 (2 × $-CH_2-N-C_3$), 70.0 (2 × $-CH_2$-N-Ar), 71.8 (C_3), 116.4 (2 × C), 120.5, 124.7, 128.1, 128.3, 129.2, 129.7 (2 × C), 130.4 (2 × C), 132.2, 133.3, 134.9, 137.6, 137.8 (2 × C), 150.0 (Ar-C), 164.2 (C=O), 167.9 (C=N) ppm.

3-Benzylpiperidin-4-anilino-7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***8***

Yield: 35.0%, mp.: 143–145°C, R_f (ethylacetate) = 0.44. MW: 458.9. IR (KBr-disc) ν max: 3434, 2828, 2358, 1994, 1602, 1481, 1318, 1120, 742, 699 cm^{-1}. MS (APCI(+)): 459, 461 (M+1), 269, 271 (M +) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.59 (s, NH), 7.66–7.74 (m, Ar-H, 13H), 4.30 (s, C3-H), 2.74–3.04 (m, CH), 2.66–2.83 (m, $-CH_2$-Ar), 1.92-2.09 (m, $-CH_2-$, 4H), 1.40–1.55 (m, $-CH_2-$, 4H) ppm.

^{13}C-NMR (DMSO-d_6) 300K δ: 32.0 (2 × $-CH_2-$), 38.9 (2 × $-CH_2-$N), 52.5 (CH), 73.1 (C3), 123.8, 127.3, 128.3 (2 × C), 128.6, 128.7, 128.9 (2 × C), 129.3 (2 × C), 129.7 (2 × C), 129.9, 131.0, 132.2, 138.3, 138.8, 139.1 (Ar-C), 164.9 (C=O), 167.2 (C=N) ppm.

7-Chloro-3-[3,4-dihydroquinolin-1(2H)-yl]-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***9***

Yield: 66.0% mp.: 160–164°C, MF: $C_{24}H_{20}ClN_3O$. MW: 401.9. IR (KBr-disc) : 3440, 3050, 2930, 2850, 2360, 1700, 1610, 1480, 830, 740 cm^{-1}. MS (APCI(+)): 402, 404 (M+1), 384, 386 (-H_2O), 269, 271(M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K: 10.91 (s, NH), 7.72 (dd, Ar-H, *J* = 8.7 Hz), 7.47–7.55 (m, phenyl-5H), 7.37 (d, Ar-H, *J* = 8.8 Hz), 7.28 (s, Ar-H), 6.95 (d, Ar-H, *J* = 7.2 Hz), 6.86 (d, Ar-H, *J* = 7.4, 7.6 Hz), 6.52 (t, Ar-H, *J* = 7.4, 7.2 Hz), 6.22 (d, Ar-H, *J* = 8.2 Hz), 5.22 (s, C3-H), 4.09 (m, –CH–), 3.62 (m, –CH–), 2.77 (m, $-CH_2-$), 1.98 (m, $-CH_2-$) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 22.0, 28.5, 45.5 ($-CH_2-$), 78.5 (C3), 111.5, 117.5, 122.6, 122.7, 125.6 (2 × C), 128.1, 128.2, 128.3, 129.3, 129.9, 130.0 (2 × C), 130.5, 131.7, 136.9, 138.1, 148.1, (Ar-C), 165.1 (C=O), 166.3 (C=N) ppm.

3-(Benzylamino)-7-chloro-5-phenyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one ***10***

Yield: 62.0%, mp.: 144–146°C, MF: $C_{22}H_{18}ClN_3O$. MW: 375.9. IR (KBr-disc) ν max: 3430, 3335, 2975, 1705, 1580, 1330, 1095, 705 cm^{-1}. MS (APCI(+)): 276, 278 (M+1), 358, 360 (-H_2O), 269, 270 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.57 (s, NH), 7.77 (dd, Ar-H, *J* = 8.7 Hz), 7.53–7.62 (m, phenyl-5H), 7.53 (d, Ar-H, J = 8.8 Hz), 7.40–7.44 (m, amine phenyl-5H), 7.30 (s, Ar-H), 5.75 (s, $-CH_2-$), 5.11 (s, C_3–H) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 52.1 (CH_2), 74.2 (C3), 121.2, 125.8 (2 × C), 126.5, 128.3, 128.5, 129.2 (2 × C), 130.1 (2 × C), 130.3, 130.6, 130.8 (2 × C), 131.0, 134.2, 136.1, 136.9 (Ar-C), 162.1 (C=O), 167.1 (C=N) ppm.

7-Chloro-3-(methylbenzyl)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***12***

Yield: 78%, mp.: 151–153°C, R_f (ether)= 0.63. MW: 389.9. IR (KBr-disc) ν max: 3398, 3324, 3128, 2899, 2832, 1767, 1522, 1477, 1320, 1150, 683 cm^{-1}. MS (APCI(+)): 390, 391 (M+1), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 3.43 (s, CH_3), 4.21 (m, 2H, $-CH_2-$), 5.13 (s, C3-H), 6.65–6.70 (d, Ar-2H, *J* = 8.8 Hz), 7.11–7.19 (t, Ar-2H, *J* = 7.9, 8.5 Hz), 7.29 (s, Ar-H), 7.34–7.40 (d, Ar-H, *J* = 8.9 Hz), 7.47–7.58 (m, phenyl-5H), 7.68–6.73 (dd, Ar-H, *J* = 8.8 Hz), 11.59 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 66.7 ($-CH_2-$), 70.4, 71.2 (CH_3 isomers), 84.4 (C3), 117.83, 123.0, 123.1, 128.2 (2 × C), 128.5, 128.8 (2 × C), 129.2, 129.3 (2 × C), 130.2, 130.1 (2 × C), 131.7, 136.8, 138.8 (Ar-C), 166.2 (C=O), 169.8 (C=N) ppm.

7-Chloro-3-(cyclohexylamino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***13***

Yield: 33.0% mp.: 166–168°C, MF: $C_{21}H_{22}ClN_3O$. MW: 367.9. IR (KBr-disc) ν max: 3403, 3092, 3033, 2927, 2863, 2358, 1700, 1623, 1474, 1322, 695 cm^{-1}. MS (APCI(+)): 368, 370 (M+1), 350, 352 (-H_2O), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.57 (s, NH), 9.58 (s, NH), 7.74–7.79 (dd, Ar-H, *J* = 8.7 Hz), 7.43–7.63 (m, phenyl-5H), 7.40–7.43 (d, Ar-H, *J* = 8.8 Hz), 7.30–7.31 (s d, Ar-H, *J* = 2.4 Hz), 5.15 (s, C3-H), 1.07–2.22 (m, $-CH_2-$, 11H) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 24.7, 25.3, 28.6, 29.8, 30.8 ($-CH_2-$), 54.6 (–CH–), 124.4 (2 × C), 127.9, 128.0, 129.1, 130.3, 130.7, 131.9, 133.2, 137.8 (2 × C), 137.9 (Ar-C), 165.7 (C=O), 167.8 (C=N) ppm.

3-Anilino-7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***16***

Yield: 80.0%, mp.: 173–175°C, MW: 361.8. IR (KBr-disc) ν max: 3425, 3025, 3065, 2930, 1710, 1590, 1440, 1340, 1100, 730, 700 cm^{-1}. MS (APCI(+)): 362, 364 (M+1), 344, 346 (–H_2O), 269, 271 (M +) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.09 (s, NH), 7.70 (dd, Ar-H, *J* = 8.7 Hz), 7.42–7.50 (m, phenyl-5H), 7.36 (d, Ar-H, J = 8.8 Hz), 7.32 (s, Ar-H), 7.23 (t, Ar-2H, *J* = 7.7, 7.8 Hz), 7.09 (t, Ar-H, *J* = 7.6, 7.3 Hz), 6.96 (d, Ar-2H, *J* = 7.7 Hz), 4.98 (s, C3-H) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 67.4 (C3), 114.2 (2 × C), 117.9, 122.8, 125.5 (2 × C), 127.1, 128.8, 129.9 (2 × C), 131.7, 136.9, 138.0, 150.2, (Ar-C), 165.1 (C=O), 167.9 (C=N) ppm.

7-Chloro-3-(2-nitroanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***17***

The compound was purified further by using column chromatography with ether. Yield: 49%, mp.: >190°C, R_f (ether)= 0.30. MF: $C_{21}H_{15}ClN_4O_3$. MW: 406,8. IR (KBr-disc) ν max: 3349, 3279, 1702, 1590, 1527, 1469, 1316, 1297, 1114, 830, 693 cm^{-1}. MS (APCI(+)):

407 (M+1), 391, 393, 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 5.19-5.22 (d, C3-H, *J* = 7.0 Hz), 6.82-6.86 (d, Ar-H, *J* = 9.2 Hz), 7.10–7.18 (m, Ar-2H), 7.33 (s, Ar-H), 7.44–7.55 (m, phenyl-5H), 7.70–7.75 (dd, Ar-H, *J* = 8.8 Hz), 7.09–8.02 (d, Ar-H, *J* = 9.3 Hz), 7.04-7.07 (d, Ar-H, *J* = 7.1 Hz), 11.15 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 70.7 (C3), 113.2, 124.1, 126.3, 127.2 (2 × C), 128.9, 129.9, 130.3, 131.3, 132.6, 137.5, 138.2 (2 × C), 138.6, 153.2 (2 × C), 166.8 (C=O), 167.7 (C=N) ppm.

7-Chloro-3-(3-chloroanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***21***

Yield: 53%, mp.: 159–161°C, R_f (ether) = 0.31. MW: 396.3. IR (KBr-disc) ν max: 3438, 2919, 2856, 2362, 2338, 1653, 1594, 1318, 1014, 671 cm^{-1}. MS (APCI(+)): 396, 397, 398 (M+1), 378, 379, 380 (−H_2O), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 5.05 (s, C3-H), 6.80–6.60 (d, Ar-H, *J* = 8.5 Hz), 6.64–6.69 (d, Ar-H, *J* = 8.0 Hz), 6.80 (s, Ar-H), 7.05–7.11 (t, Ar-H, *J* = 8.1 Hz), 7.32 (s, Ar-H), 7.33–7.35 (d, Ar-H, *J* = 8.7 Hz), 7.43–7.53 (m, phenyl-5H), 7.68–7.73 (dd, Ar-H, *J* = 8.8 Hz), 11.06 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 67.9 (C3), 117.5, 118.1, 122.6, 122.8, 125.8 (2 × C), 128.4, 128.7, 130.1, 130.5 (2 × C), 130.8, 130.9, 132.0, 133.5, 136.1, 137.2, 142.5 (Ar-C), 164.2 (C=O), 168.1 (C=N) ppm.

7-Chloro-3-(3-methoxyanilino)-5-phenyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one ***24***

Yield: 45%, mp.: 153–155°C. MF: $C_{22}H_{18}ClN_3O_2$. MW: 391.9. IR (KBr-disc) ν max: 3445, 3210, 3080, 2940, 1690, 1520, 1495, 1230, 1140, 1030 cm^{-1}. MS (APCI(+)): 392, 394 (M+1), 374, 376 (-H_2O), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.20 (s, NH), 7.65 (dd, Ar-H, *J* = 8.8 Hz), 7.43–7.51 (m, phenyl-5H), 7.31 (s, Ar-H), 7.30 (d, Ar-H, *J* = 8.7 Hz), 6.98 (t, Ar-H, 8.0, 8.0 Hz), 6.45 (d, Ar-H, *J* = 7.5 Hz), 6.27 (s, Ar-H), 6.22 (m, Ar-H), 4.89 (d, C3-H, 7.5 Hz), 3.66 (s, OCH_3) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 55.7 (OCH_3), 66.9 (C3), 104.5, 107.9, 113.0, 122.7, 125.7 (2 × C), 128.0, 128.5, 129.8 (2 × C), 129.9, 130.8, 131.5, 137.1, 138.2, 142.1 (Ar-C), 160.1 (Ar-O) 164.1 (C=O), 167.9 (C=N) ppm.

7-Chloro-3-(4-methoxyanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***25***

Yield: 62%, mp.: 163–165°C, R_f (ether) = 0.38. MW: 391.9. IR (KBr-disc) ν max: 3426, 3193, 3058, 2935, 1687, 1519, 1476, 1320, 1220, 698 cm^{-1}. MS (APCI(+)): 392, 394 (M+1), 374, 376 (-H_2O), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 3.77 (s, OCH_3), 4.80 (s, C3-H), 7.00–7.05 (d, Ar-2H, *J* = 7.8 Hz), 7.28-7.31 (d, Ar-H, *J* = 7.7 Hz), 7.32-7.36 (d, Ar-H, *J* = 7.9 Hz), 7.46-7.55 (m, phenyl-5H), 7.63–7.68 (dd, Ar-H, *J* = 8.8 Hz), 10.16 (s, NH), 10.85 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 53.3 (OCH_3), 68.1 (C3), 116.3 (2 × C), 117.2 (2 × C), 122.3, 124.5 (2 × C), 124.9, 126.6, 126.9, 129.8 (2 × C), 129.9, 130.6, 137.0, 137.4, 139.7, 153.6, 165.3 (C=O), 167.1 (C=N) ppm.

7-Chloro-3-(3,4-dimethoxyanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***27***

Yield: 50%, mp.: 194–196°C. MW: 421.9. IR (KBr-disc) ν max: 3450, 3215, 3070, 2940, 1695, 1515, 1495, 1230, 700 cm^{-1}. MS (APCI(+)): 422, 426 (M+1), 404, 406 (−H_2O), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 11.15 (s, NH), 7.70 (dd, Ar-H, *J* = 8.7 Hz), 7.43–7.48 (m, phenyl-5H), 7.36 (s, Ar-H), 7.33 (d, Ar-H, *J* = 8.8 Hz), 6.80 (d, Ar-H, *J* = 8.7 Hz), 6.19 (dd, Ar-H, *J* = 8.7 Hz), 6.06 (d, Ar-H, *J* = 7.1 Hz), 5.97 (s, Ar-H), 5.03 (d, C3-H, J = 7.1 Hz), 3.82 (s, OCH_3), 3.61(s, OCH_3) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 55.1, 65.5 (OCH_3), 67.5 (C3), 105.1, 111.7, 122.9, 125.4 (2 × C), 128.1, 128.6, 129.9 (2 × C), 130.0, 130.8, 132.1, 136.5, 137.1, 140.2 (Ar-C), 152.7, 153.1 (Ar–O–), 165.2 (C=O), 165.9 (C=N) ppm.

7-Chloro-3-(2,4-dimethylanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***34***

Yield: 67%, mp.: 158–161°C, R_f (ether) = 0.44. MF: $C_{23}H_{20}ClN_3O$. MW: 389.9. IR (KBr-disc) ν max: 3436, 3182, 2919, 2618, 1690, 1606, 1473, 1222, 1147 cm^{-1}. MS (APCI(+)): 390, 392, (M+1), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 2.32 (s, CH_3), 2.78 (s, CH_3), 4.80 (s, C3-H), 7.08–7.18 (m, Ar-2H), 7.22–7.23 (s,d Ar-H, *J* = 2.5 Hz), 7.28-7.31 (d, Ar-H, *J* = 8.5 Hz), 7.32–7.35 (d, Ar-H, J = 7.5 Hz), 7.46–7.53 (m, phenyl-5H), 7.63–7.68 (dd, Ar-H, *J* = 8.7, 8.8 Hz), 10.18 (s, NH), 10.84 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 17.5 (CH_3), 21.0 (CH_3), 83.2 (C3), 123.8 (2 × C), 125.8, 127.2, 127.8, 128.1, 128.7, 129.0 (2 × C), 129.4, 129.9, 131.2, 132.3, 132.4, 132.5, 138.2, 138.4 (Ar-C), 163.5 (C=O), 170.1 (C=N) ppm.

7-Chloro-3-(methylanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***36***

Yield: 85%, mp.: 163–165°C, R_f (ether) = 0.59. MW: 375.9. IR (KBr-disc) ν max: 3430, 3216, 3129, 2923, 2851, 2358, 1708, 1596, 1496, 1318, 114, 693 cm^{-1}. MS (APCI(+)): 376, 378 (M+1), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 3.40 (s, CH_3), 5.24 (s, C3-H), 6.68–6.71 (d, Ar-2H, *J* = 8.8 Hz), 7.12-7.18 (t, Ar-2H, *J* = 7.3, 8.5 Hz), 7.28 (s, Ar-H), 7.34–7.38 (d, Ar-H, J = 8.8 Hz), 7.48–7.55 (m, phenyl-5H), 7.68–6.73 (dd, Ar-H, J = 8.7 Hz), 11.89 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 70.1, 70.8 (CH_3 isomers), 83.4 (C3), 117.8, 123.2, 123.3, 128.2 (2 × C), 128.3, 128.9 (2 × C), 129.4, 129.4 (2 × C), 130.0, 130.3 (2 × C), 131.7, 136.9, 138.1 (Ar-C), 165.1 (C=O), 169.4 (C=N) ppm.

7-Chloro-3-(3-dimethylanilino)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one ***37***

Yield: 39%, mp.: 163–164°C, R_f (ether) = 0.66. MF: $C_{23}H_{20}ClN_3O$. MW: 389.9. IR (KBr-disc) ν max: 3420, 2925, 1700, 1600, 1481, 1320, 1121, 699 cm^{-1}. MS (APCI(+)): 390, 392, (M+1), 269, 271 (M+) m/z. ^{1}H-NMR (DMSO-d_6) 300K δ: 2.22 (s, CH_3), 3.25 (s, N-CH_3), 5.61 (s, C3-H), 7.00–7.06 (t, Ar-H, *J* = 7.8, 7.9 Hz), 7.23 (s, Ar-H), 7.22–7.30 (d, Ar-H, *J* = 7.4 Hz), 7.31 (s, Ar-H), 7.37–7.40 (d, Ar-H, *J* = 8.7 Hz), 7.49–7.56 (m, phenyl-5H), 7.64–7.66 (dd, Ar-H, J = 8.8 Hz), 7.69–7.74 (dd, Ar-H, *J* = 8.7 Hz), 10.89 (s, NH) ppm. ^{13}C-NMR (DMSO-d_6) 300K δ: 22.0 (CH_3), 58.6 (N–CH_3), 71.8 (C3), 122.3, 124.6 (2 × C), 125.2, 125.7, 127.0, 127.6, 128.6, 128.1, 128.9, 129.0, 129.3, 129.8 127.2, 127.8, 128.1, 128.7, 129.0, 129.3, 129.8 (2 × C), 129.9, 138.4, 138.4, 138.6, 149.5 (Ar-C), 165.1 (C=O), 169.4 (C=N) ppm.

Biology

Cholecystokinin binding assay, [^{125}I]I-CCK-8 receptor binding assay

CCK_1 and CCK_2 receptor binding assays were performed, by using guinea pig cerebral cortex (CCK_2) or rat pancreas (CCK_1). Male guinea pig brain tissues were prepared according to the modified method described by Saita *et al.* [33]. Pancreatic membranes were prepared as described by Charpentier et al. [34].

Tissues were homogenised in cold sucrose (0.32 M, 25 mL) for 15 strokes at 500 rpm and centrifuged at 13 000 rpm for 10 min.

The supernatant was re-centrifuged at 13 000 rpm for 20 min. The resulting pellet was re-dispersed to the required volume of buffer at 500 rpm and stored in aliquots. Binding was achieved using radioligand ^{125}I-Bolton-Hunter labeled CCK, NEN at 25 pM. The samples were incubated with membranes (0.1 mg/mL) in 20 mM Hepes, 1mM EGTA, 5 mM $MgCl_2$, 150 mM NaCl, at pH 6.5 for 2 h at RT and then centrifuged at 11 000 rpm for 5 min. The membrane pellets were washed twice with water and the bound radioactivity was measured in a Packard Cobra Auto-gamma counter (B5005; Perkin Elmer, Beaconsfield, UK). All binding assays were carried out with L-363, 260 as an internal non-specific control.

Animal studies

Experiments were conducted in male IRC mice obtained from the Animal House, Faculty of Medicine, Khon Kaen University. Each experimental group consisted of 8 animals and the treatment procedures were approved by the ethical committee, Faculty of Medicine, Khon Kaen University. Mice were intraperitoneally injected with the test compound, dissolved in 5% DMSO at the volume not more than 0.2 mL/animal. At 30 min after treatment, animals were tested as described in the following sections.

Anxiolytic activity tests

The light/dark box

Mice were placed in the light part of the light / dark box. The box was a plexiglass cage, 25 × 50 × 20 cm, having one-third as a dark and two-third as a light compartment. A 40-W light bulb was used and positioned 10 cm above the centre of the light component. The animals could walk freely between dark and light parts through the opening. The time animals spent in light part during the 5 min interval was recorded. The mouse was considered to be in the light part when its four legs were in the light part.

The elevated plus-maze

The wooden elevated plus-maze consisted of two open arms (30 × 10 cm) without any walls, two enclosed arms of the same size with 5 cm high side walls and end wall, and the central arena (10 × 10 cm) interconnecting all the arms. The maze was elevated approximately 30 cm height from the floor. At the beginning of the experiment the mouse was placed in the central arena facing one of the enclosed arms. During a 5-min interval, the time animals spent in the open arms of the plus-maze was recorded. The mouse was considered to be in the open part when it had clearly crossed the line between the central arena and the open arm with its four legs.

Nociception tests

The thermal tail-flick test

The thermal response latency was measured by the tail flick test. The animals were placed into individual restraining cages leaving the tail hanging freely. The tail was immersed into water preset at 50°C. The response time, at which the animal reacted by withdrawing its tail from water, was recorded and the cut-off time was 10 s in order to avoid damaging the animal's tissue.

The hot plate test

Mice were placed on a hot plate that was thermostatically maintained at 50°C. A plexiglass box was used to confine the animal to the hot plate. The reaction time of each animal (either paw licking or jumping) was considered a pain response. The latency to reaction was recorded. For prevention of heat injury, the cut-off time of the test was 30 s.

Antidepressant tests

The tail suspension test

Mice were hung by their tail on the tail hanger using sticky tape for tail fixation, at approximately 1 cm from the end. The hanger was fixed in the black plastic box (20 × 20 × 45 cm) with the opening at the top front. The distance between the hanger and the floor was approximately 40 cm. The mouse was suspended in the air by its tail and the immobile time was recorded during the period of 5 min. The duration of immobility was defined as the absence of all movement except for those required for respiration.

The forced swim test

The forced swim test was carried out in a glass cylinder (20 cm diameter, 30 cm height) filled with water to the height of 20 cm. The water temperature was approximately 25–28°C. Mice were gently placed into the water and the immobility time was recorded by an observer during the period of 5 min. Immobility was defined as absence of all movement and remained floating passively in the water with its head just above the water surface.

Motor activity tests

The rota-rod test

Mouse was placed on the rotating drum with the acceleration speed (Accelerator: Rota-rod, Jones & Roberts, for mice 7650, Ugo Basile, Italy). The time the animal spent on the rod is recorded.

The wire mesh grasping test

Mouse was placed on a wire mesh (20 × 30 cm). After a few seconds, the mesh was turned 180°C and the time the animal hold on the mesh was recorded.

Statistical methods

The data were expressed as mean + SD and one-way analysis of variance (ANOVA) and supplementary Tukey test for pair-wise comparison were tested to determine for any significant difference at $p < 0.05$.

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