Journal of Molecular Structure 1269 (2022) 133735

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstr

Further and new target-based benzimidazole anthelmintics active against *Teladorsagia circumcincta*

Nerea Escala[a,1], Elora Valderas-García[b,c,1], María Álvarez Bardón[b], Verónica Castilla Gómez de Agüero[c,d], José Luis López-Pérez[a,e], Francisco A. Rojo-Vázquez[d], Arturo San Feliciano[a,f], María Martínez-Valladares[c,d,**], Rafael Balaña-Fouce[b,*], Esther del Olmo[a,*]

[a] *Departamento de Ciencias Farmacéuticas: Química Farmacéutica, Facultad de Farmacia, Universidad de Salamanca, CIETUS, IBSAL, Salamanca 37007, Spain*
[b] *Departmento de Ciencias Biomédicas, Facultad de Veterinaria, Universidad de León, León 24071, Spain*
[c] *Instituto de Ganadería de Montaña, CSIC-Universidad de León, Grulleros, León 24346, Spain*
[d] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, León 24071, Spain*
[e] *Facultad de Medicina, Universidad de Panamá, Panamá*
[f] *Programa de Pós-graduaçao em Ciências Farmacêuticas, Universidade do Vale do Itajaí, UNIVALI. Itajaí, SC, Brazil*

ARTICLE INFO

Article history:
Received 6 June 2022
Revised 14 July 2022
Accepted 15 July 2022
Available online 19 July 2022

Keywords:
Benzimidazole derivatives
Teladorsagia
Anthelmintic
Docking
Structure-activity relationships

ABSTRACT

Helminth infections are one of the most prevalent parasitic diseases affecting animals and humans worldwide. Anthelmintic resistance to the main drugs used to control these infections in animals, especially ruminants, is a major global problem that needs urgent solutions. The purpose of this study was to design and obtain new benzimidazole (BZ) derivatives to evaluate their *in vitro* ovicidal and larvicidal activities. Based on previous results from 2-phenylbenzimidazoles and new docking studies of different BZ-containing scaffolds in *Teladorsagia circumcincta* tubulin four structural groups of BZs were selected. In addition to several new members of those previously reported 2-phenylBZs (type I), some twenty 2-aminoBZ derivatives (type II) and twenty-five BZ amides (types III and IV) were prepared and evaluated for their ability to inhibit egg hatching and larval motility of *T. circumcincta* with results superior to those previously achieved. Nine of the fifty-five BZs tested displayed ovicidal activity higher than 90%, and fourteen induced more than 30% larval death in the assays at 50 μM. The benzamide BZ **42** showed the best ovicidal EC_{50} value of 0.92 μM with a selectivity index > 100 respecting HepG2 cells, while the benzylamine BZ **13** attained a higher than 50% larvicidal activity. Notably, the amide BZ **39** displayed both ovicidal (100%) and larvicidal (>50%) activities. The SAR analysis and the docking studies carried out led to the conclusion that, in addition to the effects on tubulin, pending experimental confirmation, there must be other mechanisms by which the evaluated BZs prevent hatching and limit the mobility of the parasitic larvae.

Introduction

Helminthic infections are a type of parasite infection that affects both human and animal species. In humans, these parasites are classified as soil transmitted helminths and affect more than a quarter of the world's population, causing substantial disease and disability [1]. In grazing ruminants, one of the most prevalent helminth parasites worldwide are gastrointestinal nematodes (GINs), which produce important economic losses mainly derived from decreased production and increased healthcare costs [2].

During recent decades, control strategies against GINs in livestock have been mainly focused on massive drug administration which has led to the development of high levels of anthelmintic resistance all over the world [3–5]. This problem is seriously aggravated by the reduced number of types of anthelmintic drugs available on the market, requiring the development of new drugs and formulations for the treatment of these diseases.

* Corresponding author at: Departamento de Ciencias Farmacéuticas: Química Farmacéutica, Facultad de Farmacia, Universidad de Salamanca, CIETUS, IBSAL, Salamanca 37007, Spain
** Corresponding author at: Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, León 24071, Spain.
E-mail addresses: mmarva@unileon.es (M. Martínez-Valladares), rbalf@unileon.es (R. Balaña-Fouce), olmo@usal.es (E. del Olmo).
[1] Both authors shared the first position.

https://doi.org/10.1016/j.molstruc.2022.133735

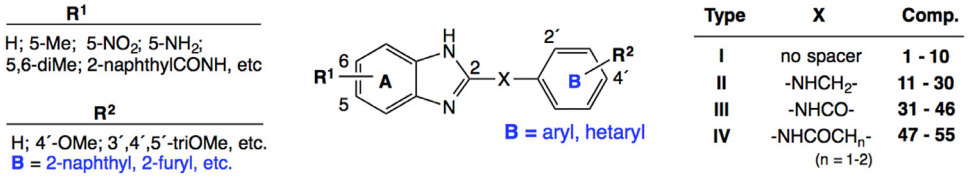


Fig. 1. General structures of types I–IV of the studied benzimidazoles.

In this context, different approaches have been proposed, such as the synthesis of novel chemical entities, drug repurposing, identification of active natural products or the synthesis of new derivatives [6]. The latter approach is a reasonable way of improving the properties and effectiveness of known drug scaffolds, as in the case of benzimidazoles (BZs), one of the most important classes of currently used anthelmintic agents. This well-known family of drugs includes good ovicidal and larvicidal agents, but they also present problems such as low water solubility, which limit their therapeutic effectiveness. Therefore, the preparation of new derivatives has been useful to develop soluble drugs, as in the case of mebendazole nitrate [7].

Previous studies of our research groups on 2-phenylbenzimidazole derivatives showed considerable ovicidal activity in GIN, *Teladorsagia circumcincta* infected sheep of compound 2-(4-bromophenyl)−1*H*-benzimidazole (EC_{50} = 6.30 μM) on a strain susceptible to albendazole, and that of compound 5-chloro-2-(4-chlorophenyl)−1*H*-benzimidazole (EC_{50} = 11.0 μM) on an albendazole-resistant strain of the same species [8]. The mechanism of action of BZs was associated with their high-affinity selective binding to the β-tubulin of the parasite, preventing the formation of microtubules, leading to the destruction of the cellular structure and the death of the parasite [9]. According to the reported docking results, the phenylBZs studied by Escala et al. [8] frequently established a hydrogen bond with the peptide carbonyl of Val236 through their BZN-H fragment acting as H-donor. Additionally, some of the most potent BZs presented π-stacking (T-shaped) aromatic ring interactions between the BZ moiety and the β-tubulin Phe200 residue.

Bearing these results in mind, and with the aim of going ahead to improve the potency and selectivity of BZ anthelmintic agents active against GIN, a planned virtual docking screening involving dozens of new structures and having different BZ arrangements and diverse functional groups, was carried out. The analysis of the results of such screening allowed discovery of some additional BZ-tubulin interactions, that led to the selection of three additional BZ scaffolds and the design of the compounds to be prepared and tested, as being described here.

2. Results and discussion

2.1. Chemistry

2.1.1. Design, synthesis and characterisation

As mentioned above, to the previous group of 2-phenylBZs (type I) three additional groups of BZ analogues derived from docking studies were added. They contain a nitrogen-based spacer of two (N-C, types II and III) or three (N-C-C, type IV) atoms between the main BZ (A) moiety and the aromatic fragment B, as represented in Fig. 1.

According to the preliminary docking results with predicted docking-score values better than −9 kcal/mol, the new compounds of type I (2-phenyl/aryl/hetarylBZs) to be prepared should contain, at the BZ fragment, any substituent able to establish additional favourable interactions with tubulin. To attain a similar or better score level, compounds of types II–IV would be guanidine (2-aminoimidazole) derivatives and should have a functional two- or three-atoms spacer between the aromatic systems A and B (Fig. 1). More than one hundred new BZ-containing structural entities including tautomers and protonated species, in addition to some of the previously reported BZs, were virtually docked in the modelled *T. circumcincta* tubulin [8], XP score values from GLIDE docking [10] found for 2-aminoBZs (type II) and 2-carbamoylBZs (types III and IV) attained levels better than −10 kcal/mol, ahead of those previously obtained for most type I BZs (see Table ST1 in Supporting Information). The increase in the scores was associated to some additional interactions found. Thus, with respect to the BZNH - Val236 H-bond and the π-stacking BZ - Phe200 interaction generally observed for the 2-phenylBZs of type I, those of type II – IV BZs complexes showed the additional H-bond donor interaction of the imidazole unit to the Glu198 residue of β-tubulin, which surely would contribute to increase the interaction energy and the docking score.

Therefore, some fifty BZ derivatives belonging to the four different types I - IV (Fig. 1) were synthesised, new and known compounds are indicated at the bottom of **Tables 1–3**. Compounds type I were prepared by known procedures from substituted *o*-phenylenediamines and aryl-aldehydes, while in the case of types II, III and IV of BZs, the 2-aminoBZ intermediates (BZ*i*) had to be previously prepared, and then condensed either, with the corresponding aldehydes followed by reduction to obtain type II 2-aminoBZs **11**–**30**, or with acids to obtain types III (**31**–**47**) and IV (**47**–**55**) of BZ amides.

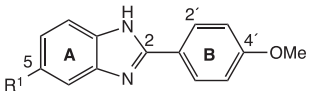
Type II compounds have been reported as potential inhibitors of 12-lipoxygenase [11], and 5-lipoxygenase[12], as well as anti-*Helicobacter pylori* agents [13], anticancer [14], or as antiproliferative [15]. With respect to compounds types III and IV, descriptions on activities such as fungicidal [16–20], antimalarial, anti-*Trypanosoma brucei* [21], anthelmintic [22], against HIV-1 proteases, or hepatitis B virus [23], neuroprotective agents targeting mGluR5 [24], inhibitors of interleukin-1-receptor-associated kinase-4 [25] antiproliferative [26], or inhibitors of 5-lipoxygenase [12] can be found.

BZs **1** to **10** have in common a 4-methoxy group attached to ring B, and BZs **5** to **10** show different substitutions on the amino group at position C-5 of the BZ nucleus. BZs **1** and **2** were obtained in our previous work [8]. The nitrophenylBZ **3** was obtained by a single step condensation of 4-nitrobenzene-1,2-diamine with 4-methoxybenzaldehyde, in the presence of sodium metabisulfite, giving a yield of 75%, which was higher than the previously attained (65%). Reduction of BZ **3** with H_2 / Pd-C provided the aniline BZ **4** with a yield of 90% (Scheme 1), which was used to prepare BZs **5** to **10**.

The treatment of BZ **4** with di-*tert*-butyl dicarbonate (Boc_2O) gave the carbamate **5** a yield of 65%. The IR spectrum of BZ **5** showed absorption bands at 1693, 1391, 1366, 1250 and 1158 cm^{-1} of *tert*-butoxycarbonyl (Boc) fragment, and at 835 cm^{-1} of the *p*-substituted ring B. The ^{1}H NMR spectrum[1] of BZ **5** displayed with

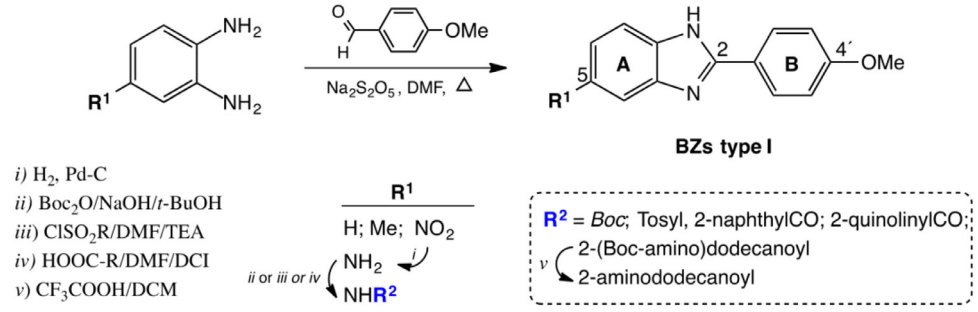
[1] *Numbering priority:* To avoid confusion in spectral assignments through the sections, and to facilitate structural comparisons between the four types of BZs, the numbering priority has been attributed first, to the benzimidazole system (A), sec-

Table 1
Inhibition of egg hatching and larval migration of *Teladorsagia circumcincta* by type I BZ derivatives.



BZ	R [1]	Inhibition % (at 50 μM) Egg Hatching (EH)	Larval Migration (LM)
1	H	**100**[a]	11.5
2	Me	**100**[a]	**40.9**[a]
3	NO_2	3.2[a]	14.7
4	NH_2	< 1[a]	6.7
5	BocNH	< 1	8.7
6	TosylNH	< 1	**48.9**
7	2-NaphthylCH_2CONH	< 1	**43.8**
8	2-QuinolinylCONH	< 1	**38.7**
9	2-Boc-aminododecanoyl-NH	< 1	**39.1**
10	2-aminododecanoyl-NH	1.4	13.2
TBZ		**100**	*nd*
LEV		*nd*	**100**

Compounds **1** to **4** are known, while **5** to **10** are new. Boc = *tert*-butoxycarbonyl; *nd*: not determined
[a] Results taken from a previous study;[8] TBZ: thiabendazole; LEV: levamisol. EHI values >90% and LMI values >25% are bolded for comparison purposes.



Scheme 1. Procedures for the synthesis of type I 2-methoxyphenylBZ derivatives.

respect to BZ **4** one intense singlet signal at 1.49 ppm that integrates for 9H and corresponds to the *tert*-butyl group, which resonate in ^{13}C NMR at 152.70, 80.34 and 28.8 ppm.

BZ **6** was obtained by tosylation of the aniline BZ **4** in 65% yield. Its IR and NMR spectral data reported in Section 4.1.2.4.1. confirm the sulfonamide formation and the incorporation of the *p*-tosyl group.

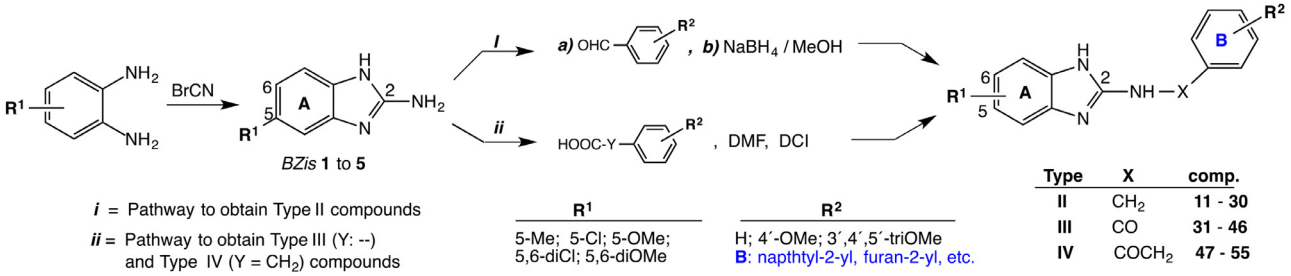
The amides BZs **7, 8** and **9** were obtained by coupling of BZ **4** with the corresponding acids in the presence of 1,1′-carbonyldiimidazole (CDI), with a yield of 67–94%, and the aminoamide BZ **10** by removal of the Boc-derivative **9** with trifluoroacetic acid. The 2-Boc-aminolauric acid used for the synthesis of BZ **9** was previously prepared by a reported procedure [27]. The ^{1}H and ^{13}C-NMR spectra of BZs **7** to **10** (Section 4.1.2.5) displayed the additional signals expected for the 2-naphthylmethyl, 2-quinolinyl, 1-Boc-aminoundecyl and 1-aminoundecyl groups, respectively.

To synthesise those type II–IV BZ derivatives, the corresponding BZ*is* had to be previously prepared by reaction of the corresponding 1,2-phenylenediamine with cyanogen bromide in aqueous methanol [28], which led to BZ*is* **1**–**5** at a yield of 62–85%. The physicochemical properties and spectral data of the resulting compounds were in accordance with the proposed structures (Section 4.1.2.7).

Type **II** BZs were obtained by a two-steps procedure (Scheme 2). BZ*is* **1**–**5** were reacted with different aldehydes in the presence of glacial acetic acid to obtain the corresponding imines, which were then reduced with $NaBH_4$ to provide BZs **11** to **30** in 17 with a 57% yield [29]. Physicochemical, MS, NMR and IR spectral data for BZs type II are reported in the experimental Sections 4.1.2.8.1-*20*. BZs **11** to **25** have a mono-substitution at position C-5 of the BZ unit with electron donating or withdrawing (Me, MeO, Cl) groups, while BZs **26** to **30** are di-substituted by either 5,6-dimethyl or 5,6-dichloro groups. Taken as an example of aminoBZs, the ^{1}H NMR of BZ **18** showed seven signals. Those corresponding to the BZ system resonate at 6.98 and 7.12 ppm as doublets (J = 7.8 Hz) for H-6 and H-7, respectively, and as a broad singlet at 7.18 for H-4. Regarding ring B, two doublets (J = 8.2 Hz) at 6.88 (H-3′+5′) and 7.32 (H-2′+6′) ppm were observed, in addition to a singlet at 3.77 of the methoxy group, and the signal at 4.56 ppm corresponding to the methylene group. In the ^{13}C NMR spectrum thirteen signals were observed, those corresponding to the B substituent at 113.98, 128.21, 131.72, 158.10 and 55.69 (methoxy group), those corresponding to the BZ system at 111.81, 118.8, 111.82, 120.11, 126.08, 134.00, 137.30 and 156.10 ppm, in addition to the signal at 45.99 ppm of the benzylic methylene.

The condensation of intermediate BZ*is* **1**–**5** with different acids in the presence of CDI, provided compounds III (BZs **31**–**46**) and IV (**47**–**55**) with yields ranging from 20% to 73% (Scheme 2). Variations in ring A included mono-substitution with electron donating or withdrawing groups (Me, MeO, Cl) and di-substitution (diMe, diCl). Variations on ring B were similar to those of type II BZs, in-

ond (with ‘) to the homo or heterocyclic system B, and third, if necessary, (with “) to the substituents attached to systems A or B. IUPAC names for all BZs are shown in the experimental section.



Scheme 2. Procedures for the synthesis of types II – IV BZs.

cluding homoaromatic systems (phenyl or naphthyl), heterocyclic (as thienyl, and pyridyl) and long-chain aliphatic substituents. BZs **47**–**53** were aryl/hetarylacetamides, while BZs **54** and **55** were lauroylamides.

Physicochemical, MS, NMR and some IR spectral data for type III and IV BZs are reported in the 4.1.2.9–11 sections. The IR spectra of these compounds showed the expected strong stretching absorption band for the amide carbonyl (NHC=O) at about 1680 cm^{-1}. ^{1}H and ^{13}C NMR spectral data for the picolinamide **42** (type III**)** and the aminophenacetamide **53** (type IV**)** are commented here. Thus, the ^{1}H NMR of BZ **42** showed eight signals, of which those corresponding to ring B resonate from 7.50 to 8.57 ppm; the ones associated to ring A resonate from 6.86 to 7.70 ppm, in addition to a singlet that integrate for three protons at 3.67 ppm ($OC\underline{H}_3$), and another at 11.83 ppm corresponding to the labile N-H protons. Its ^{13}C NMR showed fourteen signals, those of ring B resonated at 148.65, 148.17, 137.02, 127.32 and 122.73 ppm, signals associated to ring A resonate at 156.15, 147.87, 136.97, 129.93, 111.18, 110.80, 108.50 and 55.79 ppm, in addition to one at 163.54 ppm of the amide carbonyl. The ^{1}H and ^{13}C NMR spectra of BZ **53** showed the expected signals for ring A and B, with the simplification in the proton spectrum due to the double substitution on ring A, in addition to the singlet at 3.68 ppm in ^{1}H NMR and the corresponding one at 43.97 ppm in the ^{13}C spectrum of the benzylic methylene. For this compound and those type IV BZs the signal associated to the amide carbonyl appeared at 173.06 ppm, almost 10 ppm down field with respect to that of BZ 42 and those of type III.

2.2. Biological assays

2.2.1. Anthelmintic activity and selectivity

All the BZ derivatives were tested at a 50 μM concentration against a susceptible strain of *T. circumcincta* [30] and their ovicidal (egg hatching inhibition = EHI) and larvicidal (larval migration inhibition = LMI) results, determined through the corresponding assays (EHIA and LMIA), are shown in Tables 1 to 3. For those compounds showing an ovicidal activity higher than 90%, the EC_{50} values of EHI were determined, in addition to their cytotoxicity on Caco-2 and HepG2 cell cultures to calculate their selectivity indexes (Table 4).

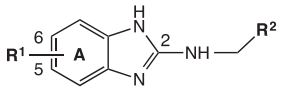
Tables 1–3 show the inhibition percentage of type I–IV BZs on *T. circumcincta* egg hatching and larval migration. The compounds listed in Table 1 are organized according to either, the size and complexity of the substituents at the C-5 (C-6) of the benzimidazole system, or to the synthetic chemical sequence of their preparation, while all of them have a 4-methoxy group attached to ring B. In the previous work, Escala et al. reported [8] on the activity results for BZs **1**–**4**, which are included here again to facilitate comparisons between new and old members of type I derivatives.

As was previously reported the 5(6)-methylBZ **2** showed an excellent ovicidal activity (100%) and fair larvicidal effects (40.9%), whereas the introduction of nitro (BZ **3**) or amino (BZ **4**) groups at such position led to the practical loss of activity. Nevertheless, based on the preliminary virtual docking predictions a small group of BZ **4** derivatives those BZs **5**–**10** were prepared and evaluated. As can be seen, these BZs did not show any ovicidal activity, but four of the six new compounds showed good larvicidal activity, and two of them, the tosyl and naphthylamides BZs **6** and **7** substantially increased the larvicidal activity of their precursor BZ **4**, even surpassing that of BZ **2**, with values of 48.9% and 43.8%, respectively. As the main consequence of the results shown in Table 1, it follows that the acylation of BZ **4** with transformation of the 5-amino group into amides (BZs **6, 7** and **8**) or carbamates (BZs **5** and **9**) fairly contributed to enhance the larvicidal activity of type I BZs, reaching or exceeding the potency of those previously reported.

Type II BZs shown in Table 2 are ordered according to the number and size of the substituents, first in ring A, then in ring B. Such compounds (BZs **11** to **30**) had electron donating (5-Me, 5-MeO or 5,6-diMe) or withdrawing (5-Cl, or 5,6-diCl) substituents on ring A, while the benzene ring B were either non-substituted, mono- (4-MeO) or tri-substituted (3,4,5-triMeO), or changed by other aromatic (naphthyl) or heteroaromatic (furyl, 5-methylfuryl and thienyl) systems.

As in the case of type I BZs, none of those evaluated type II aminoBZs showed significant hatching inhibition effects; whereas, higher than 25% larval migration inhibition effects were observed for seven of the twenty aminoBZs tested. Globally, inhibition results in the 35–50% range were found for 5-methyl and 5,6-dimethyl BZs combined with phenyl (**11**), methoxyphenyl (**12** and **26**, respectively) or 2-naphthyl (**14**) groups as B systems. Similar though lower inhibition results were found for 5,6-dichloroBZ analogues **28** (phenyl) and **29** (methoxyphenyl)**,** thus revealing that electron-donating substituents at ring A seem better, while the Me/Cl isosteric character, could be playing a certain role in such similarity. The BZ analogues containing furan, thiophene and other B ring configurations gave less relevant LMI results. On the other hand, it should be noted the strong negative influence for the activity that would be associated to the 3,4,5-trimethoxyphenyl group, whose presence practically removed the activity in BZs **19, 27** and **30**. This fact was independent of the substitution pattern of ring A, and in fair contrast with the overall positive influence of the structurally close 4-methoxyphenyl group. Most surprisingly, the regioisomeric 2,3,4-trimethoxyphenyl group, present in the most potent larvicidal BZ **13** (LMI: 54.9%), should be considered as the responsible for the 18.6% and 14.6% increases of the inhibitory potency in the respective comparison with the benzylamine **11** and the 4-methoxybenzylamine **12**. Consequently, the presence of the 2-methoxy group, which determines the absence of symmetry in the substituent and most likely an important rotational and conformational restriction, would be the ultimate cause of the great contribution of such 2,3,4-trimethoxyphenyl group to the activity. This fact must certainly be investigated through the preparation and evaluation of other benzylaminoBZ derivatives having an *ortho*-methoxy substituent, either alone or combined with other groups around ring B.

Table 2
Inhibition of egg hatching and larval migration of *Teladorsagia circumcincta* by type II 2-aminoBZ derivatives.



Type II BZ

BZ-II	R [1]	R [2]	Inhibition % (at 50 μM)	
			Egg Hatching (EH)	Larval Migration (LM)
11	5-methyl	phenyl	1.1	**36.3**
12	5-methyl	4-methoxyphenyl	1.1	**40.3**
13	5-methyl	2,3,4-trimethoxyphenyl	< 1	**54.9**
14	5-methyl	2-naphthyl	< 1	**41.5**
15	5-methyl	2-furyl	< 1	22.5
16	5-methyl	2-thienyl	< 1	< 1
17	5-chloro	phenyl	< 1	4.6
18	5-chloro	4-methoxyphenyl	< 1	12.1
19	5-chloro	3,4,5-trimethoxyphenyl	< 1	1.4
20	5-chloro	2-furyl	1.7	9.2
21	5-methoxy	phenyl	< 1	10.7
22	5-methoxy	4-methoxyphenyl	1.1	14.6
23	5-methoxy	4-(1-pyrrolidinyl)phenyl	1.0	16.4
24	5-methoxy	5-methylfur-2-yl	< 1	15.8
25	5-methoxy	2-thienyl	<1	20.0
26	5,6-dimethyl	4-methoxyphenyl	< 1	**49.8**
27	5,6-dimethyl	3,4,5-trimethoxyphenyl	< 1	< 1
28	5,6-dichloro	phenyl	2.1	**31.9**
29	5,6-dichloro	4-methoxyphenyl	1.3	**26.5**
30	5,6-dichloro	3,4,5-trimethoxyphenyl	< 1	< 1
TBZ			100.0	*nd*
LEV			*nd*	100.0

Compounds **11, 12, 15** to **18, 20** to **22** and **25** are known, while **13, 14, 19, 23, 24, 26** to **30** are new. TBZ: thiabendazole; LEV: levamisol. *nd*: not determined. LMI values >25% are bolded for comparison purposes.

An evaluation of the relative influence of ring A substituents on the activity of this type of BZs can be made by comparing those compounds having the same *p*-methoxybenzyl (B) moiety. In this way, the contribution order of the substituents to LMI resulted as follows: 5,6-diMe (BZ **26**: 49.8%) > 5-Me (**12**: 40.3) > 5,6-diCl (**29**: 26.5) > 5-MeO (**22**: 14.6) > 5-Cl (**18**: 12.1), corresponding to the respective detriments of 9.5, 12.5, 11.9 and 14.6 LMI% units, related to the successive disappearance or change of substituents from **26** to **18**. A similar sequence was observed for those aminoBZs having no-substitution on the benzene ring B, as that of BZs **11** (5-Me: 36.3%), **28** (5,6-diCl: 31.9%), **21** (5-MeO: 10.7%) and **17** (5-Cl: 4.6%). These sequences reveal for type II BZs the prevalence of the electron donating nature of the substituents on ring A, combined with the probable incidence of the mutual isosteric character of methyl and chloro substituents.

Other facts that can be mentioned are the 5.2% increase in LMI produced by changing the phenyl group in BZ **11** to the 2-naphthyl in BZ **14**, as well as the fair decreases of 13.8% and more than 35%, produced by its changing to 2-furyl (BZ **15**) and 2-thienyl (BZ **16**) groups, respectively. Since the four substituents in comparison are similarly aromatic and planar, such differences in the experimental results would have some significant influence on the global size of the hydrophobic aromatic B system on the activity.

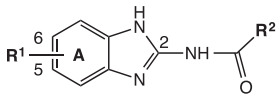
FormamidoBZ derivatives **31**–**46** (type III), and acetamido and other acylamido derivatives BZs **47**–**55** (type IV), are shown in Table 3 with the results of their evaluation. BZs in this table have the same group of substituents on ring A, with differences in those attached to ring B, and are ordered in a similar way as in Tables 1 and 2. Regarding the B nucleus, the changing of the benzene ring by that of pyridine in BZs **36, 39** and **42**, or by an aliphatic long-chain in BZs **54** and **55** can be noted. Additional changes in the substitution pattern of the benzene ring B, with electron donating or withdrawing groups at position *meta* of either mono- or di-substituted nucleus were also introduced.

As can be seen by a first glance at the results in Table 3, globally considered, type III benzamidoBZs and picolinamidoBZs showed good hatching inhibition results, with 7 out of the 16 evaluated BZs, namely benzamides **31, 37, 40**, and **43** and the picolinamides **36, 39** and **42,** exceeded the 90% ovicidal level. A second relevant fact related to egg hatching by BZ benzamides refers to the inactivity of most compounds having any substituent attached to the benzene ring B, independently of the substituents on the BZ ring A, though with the relevant exception of the methoxybenzamide BZ **38**, which attained almost 40% hatching inhibition. In terms of structure-activity relationships, it would be more interesting to compare the respective decreases of 60% and 94% of inhibition power observed for the 4′-methoxyBZs **38** and **32**, with respect to the total inhibition produced by the non-substituted BZs **37** and **31**. This fact would demonstrate the existence of a true steric hindrance around ring B in the interaction with the target.

Significantly, and in sharp contrast with type-III formamido derivatives, all type IV phenyl and thienylacetamides and the two lauroylamides tested were practically inactive against hatching of *T. circumcincta* eggs. From such results, the inefficiency of the N-C-C spacer between the BZ and ring-B units was deduced. This, combined with the above mention about the inconvenience of the substitution at the ring B of benzamides, permit the establishment of the existence of a limited distance of around 7.5 Å from the N atom of 2-aminoBZ to the end of the B moiety, whether or not substituted.

Related to larval migration inhibition by type III BZs, the only relevant larvicide was the picolinamide **39**, which is also the principal dual BZ inhibitor with LMI and EHI values of 51.2% and 100%, respectively. Another compound of this group that could be mentioned is the benzamide **33**, which although devoid of activity on eggs, showed a LMI value of 22.5%, that could be associated to its double substitution by electron-withdrawing groups if, in terms of

Table 3
Inhibition of egg hatching and larval migration of *Teladorsagia circumcincta* by BZ amides of types III and IV.



Types III and IV

BZ-III	R [1]	R [2]	Inhibition % (at 50 µM)	
			Egg Hatching (EH)	Larval Migration (LM)
31	5-methyl	phenyl	**95.5**	< 1
32	5-methyl	4-methoxyphenyl	< 1	8.8
33	5-methyl	2-chloro-5-nitrophenyl	< 1	22.5
34	5-methyl	3,5-dimethoxyphenyl	< 1	< 1
35	5-methyl	3,4,5-methoxyphenyl	< 1	6.9
36	5-methyl	2-pyridyl	**100.0**	< 1
37	5-chloro	phenyl	**100.0**	< 1
38	5-chloro	4-methoxyphenyl	39.9	< 1
39	5-chloro	2-pyridyl	**100.0**	**51.2**
40	5-methoxy	phenyl	**100.0**	15.1
41	5-methoxy	4-methoxyphenyl	< 1	< 1
42	5-methoxy	2-pyridyl	**100.0**	< 1
43	5,6-dimethyl	phenyl	**91.2**	7.2
44	5,6-dimethyl	4-methoxyphenyl	< 1	< 1
45	5,6-dichloro	Phenyl	< 1	3.6
46	5,6-dichloro	4-methoxyphenyl	< 1	3.3
BZ-IV				
47	5-methyl	3-nitrobenzyl	< 1	21.9
48	5-methyl	α-hydroxybenzyl	< 1	**34.2**
49	5-methyl	2-naphthylmethyl	< 1	2.3
50	5-methyl	2-thienylmethyl	1.00	**25.5**
51	5,6-dimethyl	3-chlorobenzyl	< 1	2.5
52	5,6-dichloro	3-nitrobenzyl	< 1	15.1
53	5,6-dichloro	3-aminobenzyl	< 1	**41.2**
54	5-methoxy	1-Boc-aminoundecyl	< 1	20.4
55	5-methoxy	1-aminoundecyl	< 1	< 1
TBZ			**100.0**	*nd*
LEV			*nd*	**100.0**

Compounds **31, 33, 35, 41, 44** and **49** are known, while **32, 34, 36** to **40, 42, 43, 45** to **48**, and 50 to **55** are new. TBZ: thiabendazole, LEV: levamisol. *nd*: not determined. EHI values >90% and LMI values >25% are bolded for comparison purposes.

Table 4
EC_{50} values of the most potent BZs against hatching of *T. circumcincta* eggs, cytotoxicity for Caco-2 and HepG2 cells and selectivity indexes (SI).

BZ	*T. circumcincta* EHI, EC_{50} (µM)	Caco-2 cells [a] (CC_{50}, µM)	HepG2 cells [a] (CC_{50}, µM)	SI [b]
31	1.51±0.03	**27.63±4.02**	**27.59±10.40**	18.3
36	1.52±0.03	7.56±1.25	**36.91±8.08**	**24.3**
37	1.58±0.03	18.24±4.23	**>50** [c]	**>31**
39	1.47±0.15	>12.50	**>25** [c]	>17
40	1.35±0.12	**61.93±32.19**	**>50** [c]	**>37**
42	**0.92±0.06**	14.35±3.40	**101.80±37.52**	**111**
TBZ [d]	**0.34±0.02**	**248.27±36.77**	**786.99±275.51**	**2315**

[a] Determined by the Alamar Blue method. [b] SI = CC_{50} (HepG2) / EC_{50} (*T.c.*). [c] Compounds with solubility problems. [d] Results for TBZ taken from a previous report.[8] Values of EC_{50} <1 µM, CC_{50} >25 µM and SI >20 are bolded to facilitate comparisons.

structure-activity relationship, it is compared with BZs **32, 34** and **35** which contain electron-donating groups.

Finally, related to LMI by acetamides and other acylamides of type IV, the inhibition values ranged from very low to suitable. Due to the diverse substitution patterns of both A and B ring systems, that makes any SAR analysis difficult, it is only worth mentioning those compounds exceeding 25% LM inhibition; namely, BZs **53** (41.2%), **48** (34.2%) and **50** (25.5%).

To obtain more precise data on the active compounds, EC_{50} values were determined for those BZs that reached inhibition levels higher than 90% in the initial EHI screening, and to establish their selectivity, their CC_{50} values of cytotoxicity for HepG2 cells (Table 4) were also determined.

As can be observed, all most potent ovicidal BZs included in Table 4 belong to type III BZ, and they are the benzamides **31, 37** and **40** and the isosteric picolinamides **36, 39** and **42**, with EC_{50} values under 1.6 µM, with BZ **42** as the most potent (0.92 µM); roughly equivalent to one-third the ovicidal potency of TBZ. Furthermore, it can be seen that all these BZs attained outstanding levels of selectivity, with BZ **42** also showing the highest index value (SI = 111). In the case of BZs **37, 39** and **40**, some solubility difficulties led to estimate their CC_{50} values as higher than 50, 25 and 50 µM, respectively.

2.3. Molecular docking, drug-likeness and toxicity risks predictions

2.3.1. Molecular docking studies

Considering the interaction of BZs with tubulin as a probable molecular mechanism for their anthelmintic effects, and aiming to find any correlation between the experimental results on eggs and larvae of *T. circumcincta* and their theoretical ability for tubulin inhibition, all BZs in this study were subjected to molecular docking with the *T. circumcincta* tubulin, as described in Section 4.1.3.

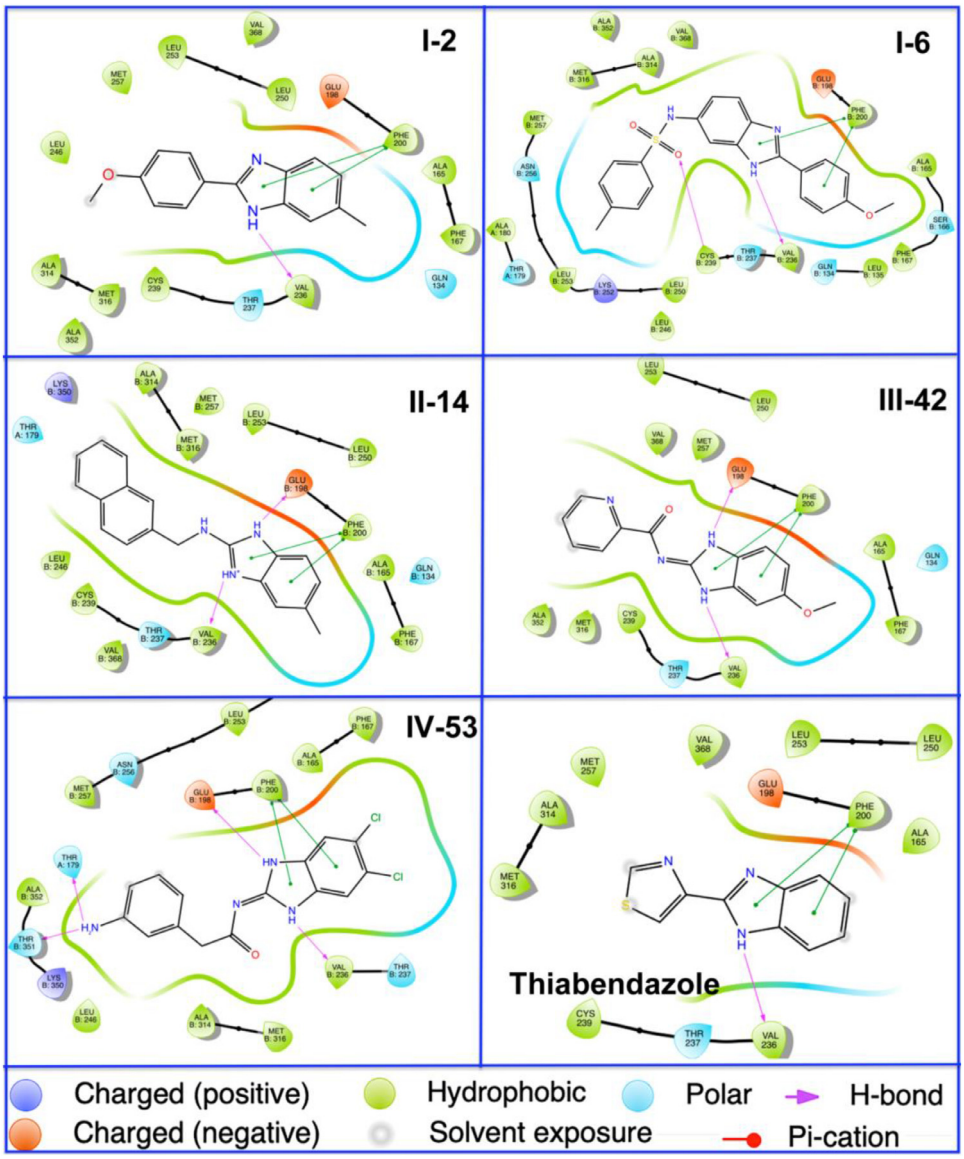


Fig. 2. 2D-maps of the docking interactions between BZs and the modelled *T. circumcincta* tubulin, with selected examples of the four BZ types, along the previously reported BZ **2** and the reference drug thiabendazole.

As in the previous study [8] the tautomerism of the BZ system was firstly examined on the four types of the compounds through a study performed with Spartan'20 (Vawefunction Inc) [10] using DFT at the RWB97X-D/6-31+G* level of theory in vacuo. The new 2-phenylBZs (Type I) behaved similarly to those previously studied, with the stability of 1H and 3H tautomers differing in some 0.5–1 kcal/mol in the presence of 5(6)-mono-substitution at ring A. The results for 2-aminoBZ derivatives (Type II) showed such equilibrium, though extended to a positively charged entity derived from protonation of the guanidine fragment in the interval of pH = 7 ± 2 applied in the study, and stabilised by charge distribution throughout the entire benzimidazole system (Scheme 3). Such a wide pH range results were appropriate in this case because *T. circumcinta* colonises the stomach and the intestine of ruminants. The BZ amides of Types III and IV showed a different behaviour with migration of the Δ^2-double bond towards the exocyclic nitrogen in most cases, surely stabilised by conjugation with the carbonyl group, and leaving two NH functions at positions 1 and 3 in such tautomer, with little difference in energy (about 1.5 kcal/mol) between the two tautomeric forms shown in the Scheme 3. These facts determine that both, the protonated amines type II and the amides III and IV with exocyclic tautomerisation, have more H-bond donors that can contribute to increasing their ability to interact with tubulin, which would potentially lead to greater affinities and more stable complexes.

The above statements are in agreement with the 2D interaction maps shown in Fig. 2, corresponding to the tubulin complexes with different BZs (additional 2D maps of some BZs are indicated in SF1 in Supporting Information). As can be seen, in addition to the BZN-H bond to Val236 residue of β-tubulin and the π-stacking interactions found for the previously reported 2-phenylBZ **2** (type I), some additional interactions can be observed for the new type I BZs and for those of types II–IV. Namely, the H-bond between de sulfonamide group of BZ **6** and the β-tubulin Cys239 residue, also the additional H-bond with Glu198 of the protonated amine **14** (type II) and of the exocyclic tautomers of BZ amides **42** (type III) and **53** (type IV).

It should also be considered that the presence of interactive functional groups in other parts of the ligand can add further positive interactions to the docking complex. Particularly, in the case

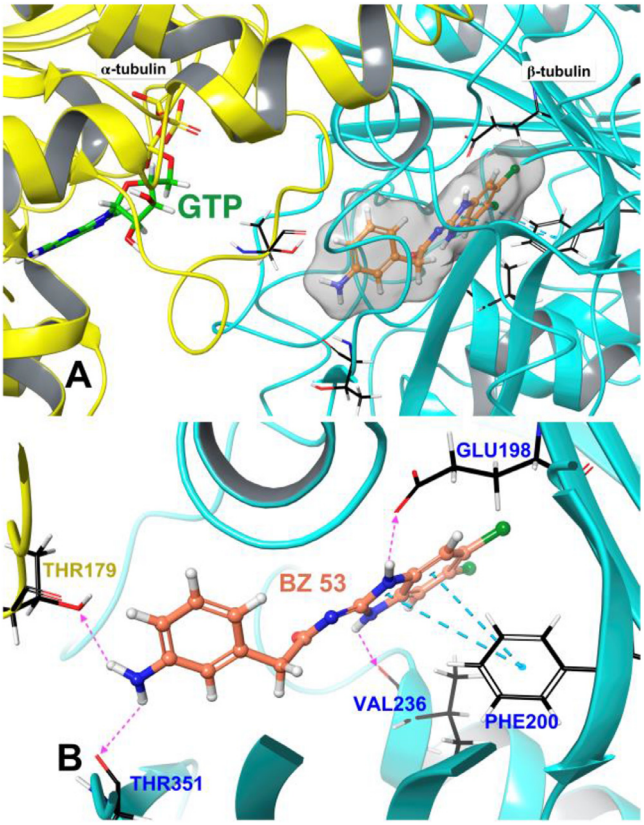


Fig. 3. (A) Docking of the amide BZ **53** into the colchicine site of the modelled *T. circumcincta* tubulin. (B) Details of the Phe200 - BZ π-stacking (T-shaped) interactions and the H-bonds of the N-H at positions 1 and 3 with Val236 and Glu198 residues of β-tubulin, as well as that between the 3-aminophenyl group and the α-tubulin (yellow) Thr179 residue, or the β-tubulin (blue) Thr 351 one.

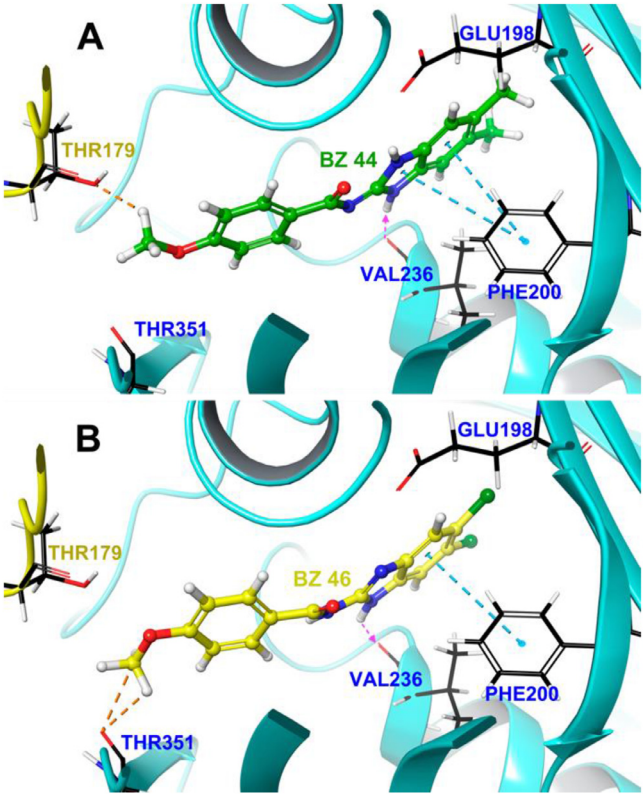


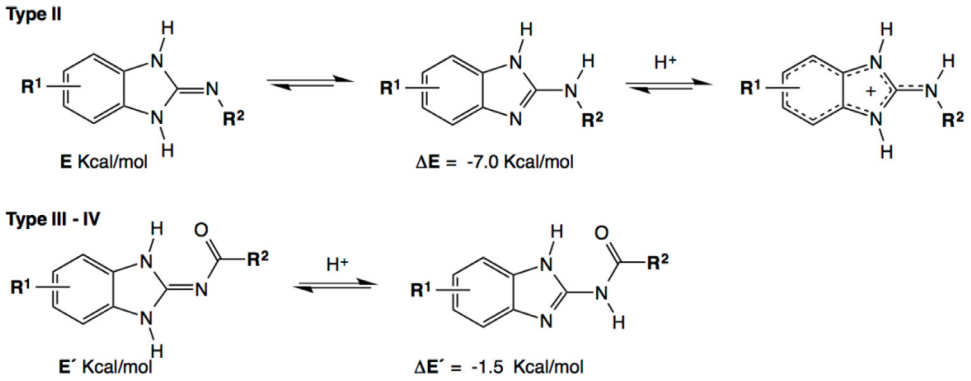
Fig. 4. (A) Docking complex of the benzamide BZ **44**, showing its bad interaction (gold broken line) with the Thr179 residue of α-tubulin, and without the usual H-bond for type III compounds between BZN-H and Glu198. (B) Similar complex of the dichloro analogue BZ **46** showing the bad interactions with the Thr351 residue of β-tubulin.

of BZ **53** the H-bonds of the *m*-amino group at the phenylacetic moiety with other β- or α-tubulin residues (Figs. 2 and 3) can be seen.

On the contrary, the presence of other substituents, although not too bulky, as in the cases of the methoxybenzamides **44** and **46** (Fig. 4), are enough to cause steric effects or bad contacts that would reduce the stability of the respective complexes (additional 3D maps of some BZs are indicated in SF2 in Supporting Information).

Interestingly, consistent with those favourable and unfavourable interactions shown in Figs. 3 and 4, the experimental LMI result for BZ **53** (41.2%) was much stronger than those found for BZs **46** (3.3%) and **44** (<1%), and such results also present a certain parallelism with the respective G-score values of −10.72, −10.42 and −9.98 predicted for the BZs (see Supporting Information, ST1).

Other aspects observed through the analysis of the BZ-tubulin complexes are related to the similarities and differences between BZs in the docking. Thus, it can be seen (Fig. 5A) that compounds of the four different types, BZs **2, 14, 34**, and **53** can dock in similar orientations into the colchicine site, whereas compounds of the same type II, as BZs **14** and **20** (Fig. 5B) are docked in practically opposed orientations. In agreement with this, they displayed very different experimental results on larval motility (LMI: 41.5 and 9.2%, respectively), and also fair differences between their predicted G-score values (−10.47 *vs* −9.20 kcal/mol), respectively (Fig. 5).



Scheme 3. Tautomeric and chemical equilibria for type II–IV BZ derivatives. Calculated generic energies (DFT RWB97X-D/6-31+G*) for BZ amines (E) and amides (E') and differences (Δ) between their respective tautomers.

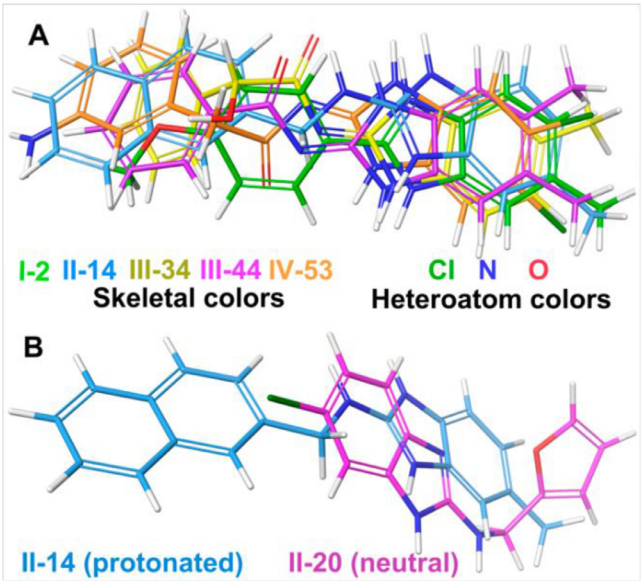


Fig. 5. (A) Superimposition of selected BZs of types I–IV as docked into the *T. circumcincta* tubulin model. (B) Different arrangement of the BZ (A) and furan (B) systems in the docked amines II–**14** and II–**20**.

Another interesting observation related to the GLIDE-Score function and the experimental activity, was that, whether on egg hatching or larval migration, the most efficient BZs presented between the best and the sufficient (−11.13 to −8.38 kcal/mol) G-Score docking values (see suppl. ST1), while commonly interacting with tubulin through their most stable tautomers or another of close stability. In apparent contrast, the current anthelmintic drug TBZ, while confirmed in this research as a very potent egg-hatching inhibitor, and having been reported to interact with the colchicine site of tubulin [31,32] (Fig. 3), attained a G-score value of −7.12 kcal/mol, substantially poorer than those mentioned above for the new active BZs. Nevertheless, it must be taken into account that TBZ is a broad-spectrum anti-infective drug, and that its tubulin polymerisation inhibition is actually complementary to the helminth-specific fumarate reductase inhibition and other biochemical mechanisms contributing to its anthelmintic action [33,34]. Something similar should happen with the new BZs, which in addition to inhibiting tubulin polymerisation, surely act through other as yet undiscovered mechanisms on the parasites.

2.3.2. Drug-likeness and toxicity risks predictions

In order to analyse the drugability of the active BZs, a virtual prediction study was performed online through the free web services SwissADME and ADMETsar platforms (see Supporting Information, ST2). To sum up, all the BZs included in Table 4 fulfil the Lipinski's Rule of Five, with MW values in the range of 251.2 and 272.7 amu (BZs **31** and **39**, respectively); clogP values between 1.48 and 2.79 (BZs **42** and **37**, respectively); H-bond acceptors between 2 and 4 (BZs **31** and **42**, respectively), and 2 H-bond donors in the six compounds. Other properties, such as their solubility above the μM level (clogS within the −3.98 to −5.14 range), and the total polar surface under 80 $Å^2$, contributed to their positive drug-likeness and lead-likeness qualifications. Most significantly, none of the active BZs in Table 4 were predicted to show any of those mutagenic, tumorigenic and irritant toxicity risks considered in the SwissADME panel.

3. Conclusions

In summary, we have synthetized and evaluated more than fifty 2-phenylbenzimidazoles, 2-(arylmethylamino)benzimidazoles, benzamides, picolinamides and other acyl derivatives of 2-aminobenzimidazoles, and a number of them exhibited promising nematocidal activities on eggs or larvae of *T. circumcincta*. Five amides of Type III displayed 100%, and two others, more than 90%, ovicidal activity on a TBZ susceptible strain at 50 μM. In addition, Ten BZs showed more than 40% larvicidal activity on such strain and at the same concentration. The amine BZ **13** was the most potent larvicide with 54.87% larval migration inhibition, while the amide BZ **39** showed both ovicidal and larvicidal activity, with an ovicidal EC_{50} value of 1.35 μM and SI value higher than 17 related to HepG2 cells. Most significantly, the amide BZ **42** resulted as the most potent and specific egg hatching inhibitor, showing the lowest ovicidal EC_{50} value of 0.92 μM and the highest SI of 110. Pending experimental confirmation, the positive results of the virtual docking of BZs into the colchicine site of a *T. circumcincta* tubulin model allowed the proposal of such biochemical interaction as partially responsible for the anthelmintic action of BZs. Indeed, although some correlations were observed between experimental activity and docking scores for small groups of compounds of every type I–IV, no true global parallelism could be found between the virtual and experimental results, which ensures that there are other mechanisms of action, yet to be discovered, that would contribute to justifying their effects on eggs and larvae of this important parasite. Finally, it can be added that two compounds with the most relevant results were selected to carry out *in vivo* preclinical efficacy and toxicity assays on gerbils, and that some of the promising results achieved will be published shortly in a specialised parasitological journal.

4. Experimental

4.1. Chemistry

4.1.1. Chemistry general

All commercial chemicals (Aldrich, Alpha, Fischer, SDS) were used as purchased and solvents (Fischer, SDS, Scharlau) purified by the standard procedures prior to use [35]. Reactions were monitored by Thin Layer Chromatography (TLC) (Kieselgel 60 F254 precoated plates, E. Merck, Germany), the spots were detected by exposure to UV light at λ 254 nm, and colourisation with 10% ninhydrin spray, and further heating of the plate. Melting points (Mp) were determined with Mel-Temp apparatus in open capillaries and were uncorrected. Separations by flash column chromatography were performed on Merck 60 silica gel (0.063–0.2 mesh). Infrared spectra were recorded on a FT-IR spectrometer Perkin Elmer System BX, using solid samples in KBr disks. NMR spectra were recorded on a Bruker Avance or Varian Mercury 400 MHz (400 MHz for ^1H, 100 MHz for ^{13}C). The spectra were measured in $CDCl_3$, methanol-d_4 or DMSO-d_6, using tetramethylsilane (TMS) as internal standard, chemical shifts (δ) are given in ppm and coupling constants (J) in Hertz (Hz). High resolution mass spectra (HRMS) were obtained by electron spray ionisation-mass spectrometry (ESI-MS) technique (5 kV) on a QSTAR XL mass spectrometer.

4.1.2. Synthesis and characterisation off BZs

4.1.2.1. Procedure for the preparation of the nitrobenzimidazole 3. A mixture of the 4-nitro-1,2-phenylenediamine (153 mg, 1.00 mmol), 4-methoxybenzaldehyde (136 mg, 1.00 mmol) and $Na_2S_2O_5$ (189 mg, 1.00 mmol) in dry DMF (4 mL) was heated to reflux for 16 h. Then, the reaction was cooled to room temperature, and water (20 mL) was added to provide a solid that was filtered off in Büchner. The solid obtained was purified by column chromatography on silica gel with *n*-hexane/ethyl acetate (8:2) as elution system and recrystallised from $CHCl_3$/methanol to obtain 205 mg (75%) of **3,** mp 237–239 °C.

*2-(4-Methoxyphenyl)−5-nitro-1*H*-benzimidazole*, **3**

Its physicochemical properties were identical as those described by Escala [8].

BZs *2-(4-Methoxyphenyl)−1*H*-benzimidazole* **1** and *2-(4-Methoxyphenyl)−5-methyl-1*H*-benzimidazole* **2** were obtained as described [8].

4.1.2.2. Procedure for the reduction of the nitrobenzimidazole 3. A solution of **3** (86 mg, 0.32 mmol) in MeOH (3 mL) in a two-necked round bottom flask was prepared, then Pd-C catalyst (3 mg) was added. Air was removed from the system, and a balloon with H_2 was placed to one of the necks. The mixture was kept at room temperature and under magnetic stirring for 2 h. Then, the Pd-C was removed by filtration through Celite, and the residue washed with MeOH. The solvent was removed in vacuo to give a red-brown solid, that was purified by crystallisation in $CHCl_3$/methanol to provided BZ **4** (69 mg, 90%), mp 196–197 °C.

*5-Amino-2-(4-methoxyphenyl)-1*H*-benzimidazole* **4**

The physicochemical properties were identical as those described by Escala [8].

4.1.2.3. Synthesis of the carbamate 5. A solution of (8 M) NaOH was added to another of BZ **4** (60 mg, 0.25 mmol) in *tert*-butanol (0.31 mL) / water (0.47 mL) (2:3) to reach pH 13. Then, 54.7 mg (0.25 mmol) of di-*tert*-butyl dicarbonate (Boc_2O) in *tert*-butanol (0.16 mL) were added and the mixture kept at room temperature for 90 min. The pH was controlled during the progress of the reaction and maintained at pH 13 by adding the necessary amounts of (8 M) NaOH. When reaction was completed (monitored by TLC), the solution was acidified to pH 5 with a solution of saturated citric acid and the mixture extracted with ethyl acetate. The organic phase was then washed with water, and dried over anhydrous sodium sulfate. Solvent was removed under vacuum, and the crude mixture purified by column chromatography with *n*-hexane/ethyl acetate (1:1) to give BZ **5** 55 mg (65% yield) as a light pink solid, mp: 148–149 °C ($CHCl_3$/methanol).

Tert-*butyl (2-(4-methoxyphenyl)-1*H*-benzimidazol)carbamate* **5**

IR (KBr) ν_{max}: 3420, 2975, 1693 (NH=CO), 1613, 1534, 1476, 1391, 1366, 1250 (C-O), 1158 (C-O), 1026, 835, 754 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (2H, d, J = 8.4 Hz, H-2′ + 6′), 7.61 (1H, d, J = 1.2 Hz, H-4), 7.37 (1H, d, J = 8.8 Hz, H-7), 7.03 (1H, dd, J_1 = 8.8; J_2 = 1.2 Hz, H-6), 6.72 (2H, d, J = 8.4 Hz, H-3′+ 5′), 3.69 (3H, s, OCH_3), 1.49 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.86 (C-4′), 152.84 (C-2), 152.70 (C<u>O</u>OC$(CH_3)_3$), 138.20 (C-7a), 137.60 (C-3a), 133.28 (C-5), 128.19 (C-2’ + 6′), 122.30 (C-1′), 115.85 (C-6), 114.18 (C-7 + 3’ + 5′), 105.70 (C-4), 80.34 (COOC<u>(</u>$CH_3)_3$), 55.21 (OCH_3), 28.39 ($(CH_3)_3$). HRMS (ESI+) *m/z* calcd. for $C_{19}H_{22}N_3O_3$ $[M + H]^+$, 340.1656, found, 340.1649.

4.1.2.4. Preparation of BZ 6. To a solution of BZ **4** (60 mg, 0.25 mmol) in THF (3.80 mL), Et_3N (0.502 mmol) was added, and then a solution of *p*-toluenesulfonyl chloride (53 mg, 0.28 mmol) in THF (2.00 mL) was added dropwise. The mixture was kept under magnetic stirring for 18 h. at room temperature and under a N_2 atmosphere. Reaction progress was monitored by TLC with *n*-hexane/ethyl acetate (2:8) as eluent. After complexion of the reaction, it was extracted with CH_2Cl_2, washed with water and the crude purified by column chromatography using *n*-hexane/ethyl acetate mixtures of increasing polarity (3:7 to 2: 8). Reaction yield in BZ **6** 55 mg (65%).

N-*(2-(4-Methoxyphenyl)-1*H*-benzimidazol-5-yl)-4-tolylsulfonamide*, **6** A white solid, mp: 196–197 °C ($CHCl_3$/methanol). IR (KBr)

ν_{max} 3342 (NH), 2924, 1611, 1520, 1455, 1430, 1296 (SO_2-N), 1254 (C–O), 1160 (SO_2-N), 1150, 1090, 1027, 966, 935, 811, 663 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 7.90 (2H, d, J = 8.0 Hz, H-2′’ + 6′), 7.35 (1H, d, J = 8.8 Hz, H-7), 7.58 (2H, d, J = 8.0 Hz, H-2’ + 6′), 7.33 (1H, d, J = 2.4 Hz, H-4), 7.17 (2H, d, J = 8.0 Hz, H-3′’ + 5′), 6.95 (2H, d, J = 8.0 Hz, H-3’ + 5′), 6.94 (1H, d, J_1 = 8.8; J_2 = 2.4 Hz, H-6), 3.76 (3H, s, OCH_3), 2.25 (3H, s, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 161.53 (C-4′), 152.92 (C-2), 143.46 (C-5), 136.80 (C-3a), 136.51 (C-7a), 136.50 (C-4′), 132.48 (C-1′), 129.08 (C-3′’+ 5′), 127.90 (C-2’ + 6′), 126.93 (C-2′’ + 6′), 121.65 (C-1′), 118.27 (C-6), 114.80 (C-7), 114.06 (C-3’ + 5′), 108.50 (C-4), 54.49 (OCH_3), 20.02 (CH_3). HRMS (ESI+) *m/z* calcd. for $C_{21}H_{20}N_3O_3S$ $[M + H]^+$, 394.1220, found, 394.1226.

4.1.2.5. General procedure for BZ 4 acylation. 0.49 mmol of 1,1′-carbonildiimidazole (CDI) was added to a solution of 0.41 mmol of the corresponding acid in dry DMF (2 mL), and the mixture was maintained at room temperature for 1 h. Then, 0.37 mmol of BZ **4** was added, and the mixture kept at room temperature with magnetic stirring for 16 h. The progress of the reaction was monitored by TLC, and *n*-hexane/ethyl acetate (1:9) was used as an eluent. After completion of the reaction, the solvent was removed under vacuum on a rotary evaporator, and the solid purified by column chromatography with *n*-hexane/ethyl acetate (2:8) as eluent. Reaction yields ranged within 67–94%.

N-*(2-(4-Methoxyphenyl)-1*H*-benzimidazol-5-yl)-2-(naphthalen-2-yl)-acetamide* **7.** A light brown solid, yield

67%, mp: 237–239 °C (methanol). IR (KBr): ν_{max} 3380, 2970, 1695, 1615, 1535, 1475, 1250, 1025, 830, 754 cm^{-1}. ^{1}H NMR (400 MHz, C_2D_6SO): δ 10.30 (1H, brs, NH), 10.20 (1H, brs, NH), 8.06 (3H, m, H-1′’ + 4′’ + 8′), 7.87 (2H, d, J = 8.4 Hz, H-2’ + 6′), 7.52 (3H, m, H-5′’+ 6′’+ 7′), 7.51 (1H, m, H-7), 7.40 (1H, m, H-3′), 7.38 (1H, m, H-4), 7.21 (1H, m, H-6), 7.07 (2H, d, J = 8.4 Hz, H-3’ + 5′), 3.83 (3H, s, OCH_3), 2.49 (2H, s, CH_2). ^{13}C NMR (100 MHz, C_2D_6SO): δ 168.72 (NHC<u>O</u>), 160.46 (C-4′), 151.21 (C-2), 140.18 (C-5), 134.29 (C-3a + 7a), 133.90 (C-1′), 133.09 (C-8a′), 131.84 (C-4a′), 127.71 (C-4′’+7′), 127.50 (C-3′’ + 8′), 127.43 (C-2’ + 6′), 126.13 (C-5′’ + 6′), 125.59 (C-2′), 122.73 (C-1′), 118.30 (C-6), 114.34 (C-3’ + 5′), 110.61 (C-4), 109.14 (C-7), 55.31 (OCH_3), 43.51 (CH_2). HRMS (ESI+) *m/z* calcd. for $C_{26}H_{22}N_3O_2$ $[M + H]^+$, 408.1707, found, 408.1707.

N-*(2-(4-Methoxyphenyl)-1*H*-benzimidazol-5-yl)quinoline-2-carboxamide* **8**

A yellow solid, yield: 94%, mp: 256–257 °C (methanol). ^{1}H NMR (400 MHz, C_2D_6SO): δ 10.78 (2H, brs), 8.62 (1H, d, J = 8.4 Hz), 8.31 (1H, m), 8.27 (1H, d, J = 8.4 Hz), 8.26 (1H, d, J = 8.4 Hz), 8.25 (2H, d, J = 7.6 Hz), 8.11 (1H, d, J = 8.4 Hz), 7.91 (1H, m), 7.75 (1H, m), 7.65 (1H, m), 7.56 (1H, m), 7.10 (2H, d, J = 7.6 Hz), 3.84 (3H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 162.40, 160.55, 151.62, 145.90, 140.80, 138.15, 133.24, 130.54, 130.61, 129.34, 128.87, 128.25, 128.10 (3C), 127.89, 122.69, 118.33 (2C), 116.33, 114.36 (2C), 110.73, 55.32. HRMS (ESI+) *m/z* calcd. for $C_{24}H_{19}N_4O_2$ $[M + H]^+$, 395.1502, found, 395.1498.

Tert-*butyl(1-((2-(4-methoxyphenyl)-1*H*-benzimidazole-5-yl)amino)-1-oxododecan-2-yl)carbamate* **9**

A light brown solid, yield: 69%, mp: 169–170 °C ($CHCl_3$/methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.99 (1H, brs, H-4), 7.93 (2H, d, J = 8.8 Hz, H-2′+6′), 7.45 (1H, d, J = 8.4 Hz, H-7), 7.25 (1H, brd, J = 8.4 Hz, H-6), 6.99 (2H, d, J = 8.8 Hz, H-3′+5′), 4.21 (1H, m, H-2′), 3.81 (3H, s, OCH_3), 1.82 (2H, m, CH_2-3′), 1.76 (2H, m, CH_2–4′), 1.45 (9H, s, $(CH_3)_3$), 1.23 (14H, m, $(CH_2)_7$), 0.84 (3H, t, J = 7.2 Hz, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 172.34 (NHC<u>O</u>), 161.47 (C-4′), 156.56 (C<u>O</u>OC$(CH_3)_3$), 152.55 (C-2), 137.97 (C-7a), 136.47 (C-3a), 133.43 (C-5), 127.87 (C-2’ + 6′), 121.76 (C-1′), 116.06 (C-6), 114.62 (C-7), 114.05 (C-3’ + 5′), 105.80 (C-4), 79.22 (COOC<u>(</u>$CH_3)_3$), 55.26 (CH-α), 54.49 (OCH_3), 32.34, 31.63, 29.29, 29.25, 29.20, 28.96, 28.46 ($(CH_3)_3$), 27.38, 25.60, 22.31, 13.06 (CH_3). HRMS (ESI+) *m/z* calcd. for $C_{31}H_{45}N_4O_4$ $[M + H]^+$, 537.3435, found, 537.3423.

4.1.2.6. Obtaining BZ 10 by deprotection of 9. 40 mg (0.074 mmol) of BZ **9** were dissolved in CH_2Cl_2 (3 mL) and kept at 0 °C under stirring. Then, trifluoroacetic acid (0.6 mL) was added, and the reaction is monitored by running TLC [eluent: *n*-hexane / ethyl acetate (1:9)] every 15 min. After the reaction has finished, it was extracted with CH_2Cl_2 and washed with water. The solvent was removed under vacuum on a rotary evaporator, and the crude purified by crystallisation [*n*-hexane / CH_2Cl_2] at 0 °C to provide BZ **10**, 10 mg in 31% yield.

*2-Amino-*N*-(2-(4-methoxyphenyl)-1*H*-benzimidazole-5-yl)dodeca namide, BZ-***10**

A brown solid, mp: 103–105 °C. IR (KBr): ν_{max} 3374 (NH), 3274 (NH), 3450, 2922, 1647 (NHCO), 1614, 1534, 1495, 1454, 1252, 1175, 1035, 830 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (1H, brs, H-4), 7.89 (2H, d, J = 8.4 Hz, H-2' + 6'), 7.45 (1H, d, J = 8.4 Hz, H-7), 6.97 (1H, d, J = 8.4 Hz, H-6), 6.81 (2H, d, J = 8.4 Hz, H-3'+5'), 3.67 (3H, s, OCH_3), 3.42 (1H, m, H-2'), 1.83 (2H, m, CH_2–3'), 1.47 (2H, m, CH_2–4'), 1.14 (14H, m, $(CH_2)_7$), 0.78 (3H, t, J = 7.2 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.54 (NHCO), 161.14 (C-4'), 152.10 (C-2), 138.40 (C-7a), 136.40 (C-3a), 133.20 (C-5), 128.03 (C-2' + 6'), 122.30 (C-1'), 119.10 (C-6), 114.38 (C-3' + 5'), 110.20 (C-7), 109.30 (C-4), 55.65 (CH-2'), 55.38 (OCH_3), 34.92, 31.88, 29.56, 29.50 (2C), 29.41, 29.30, 25.98, 22.66, 14.10 (CH_3). HRMS (ESI+) *m/z* calcd. for $C_{26}H_{37}N_4O_2$ $[M + H]^+$ 437.2911, found, 437.2903.

4.1.2.7. General procedure for the preparation of 2-aminobenzimidazole intermediates BZis (1–5). Solutions of the corresponding 1,2-phenylendiamine (0.02 mmol) and cyanogen bromide (0.03 mmol) in aqueous methanol (50% v/v) were prepared separately. Then were mixed in an Erlenmeyer flask and maintained with continuous magnetic stirring at room temperature for 96 h. The reaction was monitored by TLC with ethyl acetate as eluent. After reaction completion, the methanol was removed under vacuum, and the crude purified by column chromatography using ethyl acetate as eluent [28].

*5-Methyl-1*H*-benzimidazol-2-amine,* BZ***i* 1**

A brown solid, yield: 85%, mp: 197–198 °C (methanol). IR (KBr): ν_{max} 3415, 3380, 2920, 1648, 1567, 1487, 1277, 1223, 1106, 890, 868, 807, 798 cm^{-1}. ^{1}H NMR (CD_3OD): δ 7.04 (1H, d, J = 8.4 Hz, H-7), 6.99 (1H, brs, H-4), 6.80 (1H, dd, J_1 = 8.4; J_2 = 1.6 Hz, H-6), 2.35 (3H, s, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 155.09 (C-2), 137.82 (C-3a), 135.46 (C-7a), 129.89 (C-6), 129.47 (C-5), 111.73 (C-4), 110.99 (C-7), 20.18 (CH_3). HRMS (ESI+) *m/z* calcd. for $C_8H_{10}N_3$ $[M + H]^+$, 148.0869, found, 148.0868.

*5-Chloro-1*H*-benzimidazol-2-amine,* BZ***i* 2**

A light brown solid, yield: 85%, mp: 188–189 °C (methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.37 (1H, d, J = 2.0 Hz), 7.31 (1H, d, J = 8.8 Hz), 7.24 (1H, dd, J_1 = 8.8; J_2 = 2.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 153.57, 134.53, 131.73, 127.19, 121.76, 111.86, 111.43. HRMS (ESI+) *m/z* calcd. for $C_7H_7N_3Cl$ $[M + H]^+$, 168.0328, found, 168.0317.

*5-Methoxy-1*H*-benzimidazol-2-amine,* BZ***i* 3**

A black solid, yield: 62%, mp: 184–185 °C (methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.05 (1H, d, J = 8.8 Hz), 6.79 (1H, d, J = 2.4 Hz), 6.61 (1H, dd, J_1 = 8.8; J_2 = 2.4 Hz), 3.77 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 155.21, 155.02, 138.22, 130.76, 111.15, 107.46, 97.19, 54.89. HRMS (ESI+) *m/z* calcd. for $C_8H_{10}N_3O$ $[M + H]^+$, 164.0824, found, 164.0816.

*5,6-Dimethyl-1*H*-benzimidazol-2-amine,* BZ***i* 4**

A dark red solid, yield: 85%, mp: 189–190 °C (methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.04 (2H, s), 2.23 (3H, s), 2.20 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 150.20, 132.32, 132.01, 127.56 (2C), 111.75, 111.46, 18.75 (2C). HRMS (ESI+) *m/z* calcd. for $C_9H_{12}N_3$ $[M + H]^+$, 162.1031, found, 162.1019.

5,6-Dichloro-1H-benzimidazol-2-amine, BZ***i* 5**

A pink solid, yield: 85%, mp: 190–191 °C (methanol). ^{1}H NMR (400 MHz, C_2D_6SO): δ 8.74 (2H, s), 7.59 (NH_2). ^{13}C NMR (100 MHz, C_2D_6SO): δ 151.98, 130.24 (2C), 125.57 (2C), 113.48, 112.59. HRMS (ESI+) *m/z* calcd. for $C_7H_6N_3Cl_2$ $[M + H]^+$, 201.9939, found, 201.9933.

4.1.2.8. General procedure for the preparation of type II BZs 11 to 30 [29]. To a solution of 0.75 mmol of each BZ*i* and the same proportion of the corresponding aldehyde in 10 mL of absolute EtOH, three drops of glacial acetic acid were added. The mixture was refluxed for 22 h. at 60 °C. Then, $NaBH_4$ (2.62 mmol) was slowly added, and the mixture was heated under reflux for 1 h. Subsequently, of water (7.5 mL) was added, and the $NaBH_4$ excess was neutralised with 2–3% glacial acetic acid. The mixture was cooled to 4 °C for 2 h. in the refrigerator, and then, the solvent was removed in vacuum on a rotary evaporator to provide a precipitate that was purified by column chromatography, eluted with mixtures *n*-hexane/ethyl acetate of increasing the polarity from (1:1) to (1:9). Yields ranged within 17–57%.

N-*Benzyl-5-methyl-1*H*-benzimidazol-2-amine* **11**

A light red solid, yield: 25%, mp: 114–115 °C (methanol). IR (KBr): ν_{max} 3350 (NH), 2918, 1638, 1579, 1490, 1452, 1273, 801, 750, 695 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 7.37 (2H, d, J = 7.2 Hz, H-2' + 6'), 7.30 (2H, t, J = 7.2 Hz, H-3' + 5'), 7.22 (1H, d, J = 7.2 Hz, H-4'), 7.05 (1H, d, J = 7.6 Hz, H-7), 7.04 (1H, d, J = 1.2 Hz, H-4), 6.78 (1H, dd, J_1 = 7.6; J_2 = 1.2 Hz, H-6), 4.54 (2H, s, CH_2), 2.34 (3H, s, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 155.37 (C-2), 139.18 (C-5), 137.81 (C-3a), 135.50 (C-7a), 129.50 (C-1'), 128.11 (C-3' + 5'), 126.79 (C-2' + 4' + 6'), 120.92 (C-6), 111.76 (C-4), 111.02 (C-7), 46.05 (CH_2), 20.24 (CH_3). HRMS (ESI+) *m/z* calcd. for $C_{15}H_{16}N_3$ $[M + H]^+$, 238.1266, found, 238.1282.

N-*(4-Methoxybenzyl)-5-methyl-1*H*-benzimidazol-2-amine* **12**

A dark orange solid, yield: 45%, mp: 107–108 °C (methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.30 (2H, d, J = 8.8 Hz, H-2' + 6'), 7.07 (1H, d, J = 7.8 Hz, H-7), 7.04 (1H, d, J = 1.2 Hz, H-4), 6.89 (2H, d, J = 8.8 Hz, H-3' + 5'), 6.77 (1H, dd, J_1 = 7.8; J_2 = 1.2 Hz, H-6), 4.60 (2H, s, CH_2), 3.75 (3H, s, OCH_3), 2.36 (3H, s, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 158.73 (C-4'), 155.41 (C-2), 137.92 (C-3a), 137.14 (C-5), 135.40 (C-7a), 131.84 (C-1'), 128.38 (C-2' + 6'), 121.13 (C-6), 114.00 (C-3' + 5'), 111.80 (C-4), 111.10 (C-7), 55.80 (OCH_3), 45.39 (CH_2), 20.36 (CH_3). HRMS (ESI^+) *m/z* calcd. for $C_{16}H_{18}N_3O$ $[M + H]^+$, 268.1450, found, 268.1469

*5-Methyl-*N*-(2,3,4-trimethoxybenzyl)-1*H*-benzimidazol-2-amine* **13**

A light yellow oil, yield: 45%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.10 (1H, d, J = 8.4 Hz), 7.04 (1H, brs), 7.00 (1H, d, J = 8.8 Hz), 6.89 (1H, d, J = 8.4 Hz), 6.65 (1H, d, J = 8.8 Hz), 4.47 (2H, s), 3.85 (3H, s), 3.77 (3H, s), 3.76 (3H, s), 2.34 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 153.64, 153.49, 151.02, 141.83, 131.94, 131.34, 134.24, 123.36, 122.99, 122.49, 111.91, 111.15, 107.50, 61.22, 60.68, 55.84, 41.77, 21.34. HRMS (ESI^+) *m/z* calcd. for $C_{18}H_{21}N_3ONa$ $[M + Na]^+$, 350.1475, found, 350.1472.

*5-Methyl-*N*-(naphthalen-2-ylmethyl)-1*H*-benzimidazol-2-amine* **14**

A yellow solid, yield: 17%, mp: 179–180 °C (methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.81 (4H, m), 7.50 (1H, m), 7.41 (2H, m), 7.07 (1H, d, J = 8.0 Hz), 7.01 (1H, d, J = 1.4 Hz), 6.79 (1H, dd, J_1 = 8.0; J_2 = 1.4 Hz), 4.71 (2H, s), 2.34 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 155.38, 137.90, 136.66, 135.42, 133.53, 132.85, 129.63, 127.87, 127.37, 127.25, 125.76, 125.35, 125.28, 125.25, 121.00, 111.77, 111.05, 46.20, 20.22. HRMS (ESI^+) *m/z* calcd. for $C_{19}H_{17}N_3Na$ $[M + Na]^+$, 310.1314, found, 310.1304.

N-*(Furan-2-ylmethyl)-5-methyl-1*H*-benzimidazol-2-amine* **15**

A yellow-orange solid, yield: 20%, mp: 136–137 °C (methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 7.39 (1H, brs), 7.05 (1H, d, J = 8.0 Hz), 7.00 (1H, brs), 6.78 (1H, d, J = 8.0 Hz), 6.30 (1H, brs), 6.27 (1H, brs), 4.49 (2H, s), 2.32 (3H, s). ^{13}C NMR (100 MHz,

CD_3OD): δ 156.10, 153.66, 143.32, 138.65, 136.38, 131.15, 122.46, 113.15, 112.46, 111.32, 107.98, 40.69, 21.59. HRMS (ESI^+) *m/z* calcd. for $C_{13}H_{14}N_3O$ $[M + H]^+$, 228.1131, found, 228.1141.

5-Methyl-N-*(thiophen-2-ylmethyl)-1*H-*benzimidazol-2-amine* **16**

An orange oil, yield: 35%. IR (NaCl): ν_{max} 3412, 3375, 2915, 1645, 1567, 1530, 1487, 1223, 1106, 890, 807, 798 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 7.13 (1H, brd, J = 5.2 Hz, H-5′), 7.12 (1H, d, J = 7.2 Hz, H-7), 7.05 (1H, brs, H-4), 6.92 (1H, d, J = 3.2 Hz, H-3′), 6.86 (1H, dd, J_1 = 5.2; J_2 = 3.2 Hz, H-4′), 6.85 (1H, d, J = 7.2 Hz, H-6), 4.70 (2H, s, CH_2), 2.34 (3H, s, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 150.12 (C-2), 137.93 (C-2′), 133.79 (C-5), 129.65 (C-3a), 127.32 (C-7a), 127.04 (C-4′), 126.89 (C-3′), 125.77 (C-5′), 124.59 (C-6), 111.98 (C-4), 111.31 (C-7), 42.33 (CH_2), 21.37 (CH_3). HRMS (ESI^+) *m/z* calcd. for $C_{13}H_{14}N_3S$ $[M + H]^+$, 244.0902, found, 244.0897.

N-*Benzyl-5-chloro-1*H-*benzimidazol-2-amine* **17**

A brown-orange solid, yield: 19%, mp: 123–124 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 7.37 (2H, m), 7.32 (2H, t, J = 7.2 Hz), 7.24 (1H, m), 7.16 (1H, s), 7.11 (1H, d, J = 7.6 Hz), 6.93 (1H, d, J = 7.6 Hz), 4.56 (2H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 156.24, 138.93, 136.70, 132.10, 128.16 (2C), 126.88 (3C), 125.34, 119.76, 111.67 (2C), 45.93. HRMS (ESI^+) *m/z* calcd. for $C_{14}H_{13}N_3Cl$ $[M + H]^+$, 258.0793, found, 258.0796.

5-Chloro-N-*(4-methoxybenzyl)-1*H-*benzimidazol-2-amine* **18**

A yellow solid, yield: 40%, mp: 114–150 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 7.32 (2H, d, J = 8.2 Hz), 7.18 (1H, brs), 7.12 (1H, d, J = 7.8 Hz), 6.98 (1H, d, J = 7.8 Hz), 6.88 (2H, d, J = 8.2 Hz), 4.56 (2H, s), 3.77 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 158.10, 156.10, 137.20, 134.00, 131.72, 128.21 (2C), 126.08, 120.11, 113.98 (2C), 111.82, 111.81, 55.69, 45.99. HRMS (ESI^+) *m/z* calcd. for $C_{15}H_{15}ON_3Cl$ $[M + H]^+$, 288.0904, found, 288.0914.

5-Chloro-N-*(3,4,5-trimethoxybenzyl)-1*H-*benzimidazol-2-amine* **19**

A light orange solid, yield: 25%, mp: 109–110 °C (methanol). ^1H NMR (400 MHz, $CDCl_3$): δ 7.25 (1H, brs), 7.10 (1H, d, J = 8.0 Hz), 6.99 (1H, d, J = 8.0 Hz), 6.39 (2H, s), 4.41 (2H, s), 3.77 (3H, s), 3.63 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.73, 152.84 (2C), 137.60, 136.38, 134.20, 133.19, 126.16, 120.80, 111.94 (2C), 103.33 (2C), 60.44, 55.52 (2C), 46.63. HRMS (ESI^+) *m/z* calcd. for $C_{17}H_{19}O_3N_3Cl$ $[M + H]^+$, 328.1110, found, 348.1104.

5-Chloro-N-*(furan-2-yl-methyl)-1*H-*benzimidazol-2-amine* **20**

A dark red solid, yield: 21%, mp: 128–129 °C (methanol). IR (KBr): ν_{max} 3411, 2921, 1630, 1603, 1466, 1384, 1266, 1058, 919, 803, 695 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 7.45 (1H, dd, J_1 = 1.2; J_2 = 0.8 Hz, H-5′), 7.20 (1H, d, J = 1.6 Hz, H-4), 7.14 (1H, J = 8.0 Hz, H-7), 6.99 (1H, J_1 = 8.0; J_2 = 1.6 Hz, H-6), 6.34 (1H, dd, J_1 = 3.2; J_2 = 0.8 Hz, H-3′), 6.32 (1H, d, J = 3.2 Hz, H-4′), 4.56 (2H, s, CH_2). ^{13}C NMR (100 MHz, CD_3OD): δ 154.84 (C-2), 151.55 (C-2′), 142.21 (C-5′), 137.46 (C-3a), 134.29 (C-7a), 126.11 (C-5), 121.62 (C-6), 111.65 (C-4), 111.38 (C-7), 109.62 (C-4′), 106.94 (C-3′), 39.21 (CH_2). HRMS (ESI^+) *m/z* calcd. for $C_{12}H_{10}N_3ClO$ $[M + H]^+$, 248.0585, found, 248.0587.

N-*Benzyl-5-methoxy-1*H-*benzimidazol-2-amine* **21**

A dark orange, yield: 45%, mp: 181–182 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 7.37 (2H, dd, J_1 = 7.6; J_2 = 1.2 Hz), 7.31 (2H, dd, J_1 = 7.6; J_2 = 7.2 Hz), 7.21 (1H, dd, J_1 = 7.2; J_2 = 1.2 Hz), 7.04 (1H, d, J = 8.4 Hz) 6.80 (1H, d, J = 2.4 Hz), 6.58 (1H, dd, J_1 = 8.4; J_2 = 2.4 Hz), 4.54 (2H, s), 3.76 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 155.65, 155.05, 139.17, 138.92, 131.90, 128.11 (2C), 126.90 (2C), 126.78, 111.15, 107.12, 97.33, 54.89, 46.04. HRMS (ESI^+) *m/z* calcd. for $C_{15}H_{16}N_3O$ $[M + H]^+$, 254.1288, found, 254.1287.

5-Methoxy-N-*(4-methoxybenzyl)-1*H-*benzimidazol-2-amine* **22**

A dark yellow solid, yield: 43%, mp: 110–111 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 7.30 (2H, d, J = 8.8 Hz), 7.05 (1H, d, J = 8.4 Hz), 6.87 (2H, d, J = 8.8 Hz), 6.80 (1H, d, J = 2.4 Hz), 6.58 (1H, d, J_1 = 8.4; J_2 = 2.4 Hz), 4.56 (2H, s), 3.75 (3H, s), 3.76 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 158.83, 155.81, 155.78, 139.20, 136.00, 130.33, 128.24 (2C), 113.95 (2C), 111.99, 107.77, 97.94, 55.92, 55.13, 46.41. HRMS (ESI^+) *m/z* calcd. for $C_{16}H_{18}N_3O_2$ $[M + H]^+$, 284.1399, found, 284.1339.

5-Methoxy-N-*(4-(pyrrolidin-1-yl)benzyl)-1*H-*benzimidazol-2-amine,* **23**

A dark orange solid, yield: 40%, mp: 175–176 °C ($CHCl_3$/methanol). IR (KBr): ν_{max} 3430, 1925, 1620, 1585, 1523, 1487, 1375, 1156, 1110, 802 cm^{-1}. ^1H NMR (400 MHz, CD_3OD+$CDCl_3$): δ 7.25 (2H, d, J = 8.8 Hz, H-2′ + 6′), 7.17 (1H, d, J = 8.4 Hz, H-7), 6.97 (1H, d, J = 2.0 Hz, H-4), 6.73 (1H, dd, J_1 = 8.4; J_2 = 2.0 Hz, H-6), 6.58 (2H, d, J = 8.8 Hz, H-3′ + 5′), 4.54 (2H, s, CH_2), 3.88 (3H, s, OCH_3), 3.32 (4H, m, H-2′′+5′′), 2.10 (4H, m, H-3′′ + 4′′). ^{13}C NMR (100 MHz, CD_3OD+$CDCl_3$): δ 155.06 (C-2), 154.94 (C-5), 147.46 (C-4′), 138.23 (C-3a), 130.71 (C-7a), 128.60 (C-2′ + 6′), 124.46 (C-1′), 111.71 (C-3′ + 5′), 111.39 (C-7), 107.63 (C-6), 97.68 (C-4), 55.89 (OCH_3), 47.62 (C-2′′ + 5′′), 46.52 (CH_2), 25.34 (C-3′′ + 4′′). HRMS (ESI^+) *m/z* calcd. for $C_{19}H_{23}N_4O$ $[M + H]^+$, 323.1866, found, 323.1873.

5-Methoxy-N-*((5-methylfuran-2-yl)methyl)-1*H-*benzimidazol-2-amine,* **24**

A dark orange solid, yield: 32%, mp: 141–142 °C ($CHCl_3$/methanol). ^1H NMR (400 MHz, $CDCl_3$): δ 7.09 (1H, d, J = 8.4 Hz), 6.83 (1H, d, J = 2.4 Hz), 6.62 (1H, dd, J_1 = 8.4; J_2 = 2.4 Hz), 6.04 (1H, d, J = 2.8 Hz), 5.81 (1H, d, J = 2.8 Hz), 4.47 (2H, s), 3.74 (3H, s), 2.15 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.34, 154.45, 152.06, 149.28, 137.61, 129.97, 111.91, 108.31 (2C), 106.23, 97.72, 55.83, 40.25, 13.39. HRMS (ESI^+) *m/z* calcd. for $C_{14}H_{15}N_3O_2Na$ $[M + Na]^+$, 280.1056, found, 280.1046.

5-Methoxy-N-*(thiophen-2-yl-methyl)-1*H-*benzimidazol-2-amine,* **25**

A dark brown solid, yield: 32%, mp: 167–168 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 7.26 (1H, dd, J_1 = 4.8; J_2 = 0.8 Hz), 7.06 (1H, d, J = 8.4 Hz), 7.05 (1H, dd, J_1 = 3.6; J_2 = 0.8 Hz), 6.93 (1H, dd, J_1 = 4.8; J_2 = 3.6 Hz), 6.81 (1H, d, J = 2.8 Hz), 6.60 (1H, dd, J_1 = 8.4; J_2 = 2.8 Hz), 4.71 (2H, s), 3.77 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 156.53, 156.49, 143.75, 140.50, 133.10, 127.63, 126.39, 125.67, 112.70, 108.63, 98.63, 56.26, 42.55. HRMS (ESI^+) *m/z* calcd. for $C_{13}H_{19}N_3OS$ $[M + H]^+$, 260.0852, found, 280.0855.

N-*(4-Methoxybenzyl)-5,6-dimethyl-1*H-*benzimidazol-2-amine,* **26**

A dark yellow solid, yield: 57%, mp: 157–158 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 6.90 (2H, d, J = 8.4 Hz), 6.58 (2H, s), 6.47 (2H, d, J = 8.4 Hz), 4.06 (2H, s), 3.36 (3H, s), 1.87 (6H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 160.45, 156.08, 138.76, 136.76 (2C), 134.60, 132.30, 129.70 (2C), 114.87 (2C), 113.50 (2C), 55.63, 47.02, 20.20 (2C). HRMS (ESI^+) *m/z* calcd. for $C_{17}H_{20}N_3O$ $[M + H]^+$, 282.1606, found, 282.1606.

5,6-Dimethyl-N-*(3,4,5-trimethoxybenzyl)-1*H-*benzimidazol-2-amine,* **27**

A dark yellow solid, yield: 40%, mp: 114–115 °C ($CHCl_3$/methanol). IR (KBr): ν_{max} 3250, 3066, 1681, 1648, 1565, 1250, 1220, 1120, 890, 872, 807, 798 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 6.99 (2H, s, H-4 + 7), 6.43 (2H, s, H-2′ + 6′), 4.43 (2H, s, CH_2), 3.76 (3H, s, 4′–OCH_3), 3.62 (6H, s, 3′ + 5′–OCH_3), 2.23 (6H, s, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.62 (C-2), 153.27 (C-3′ + 5′), 136.86 (C-4′), 135.12 (C-3a + 7a), 134.27 (C-1′), 129.13 (C-5 + 6), 112.88 (C-4 + 7), 103.87 (C-2′ + 6′), 60.76 (4′–OCH_3), 55.87 (3′ + 5′–OCH_3), 47.18 (CH_2), 20.02 ((CH_3)$_2$). HRMS (ESI^+) *m/z* calcd. for $C_{19}H_{24}N_3O_3$ $[M + H]^+$, 342.1812, found, 342.1807.

N-*Benzyl-5,6-dichloro-1*H-*benzimidazol-2-amine,* **28**

A light brown solid, yield: 17%, mp: 198–199 °C ($CHCl_3$/methanol). ^1H NMR (400 MHz, $CDCl_3$): δ 7.84 (2H, s), 7.39 (2H, m), 7.27 (3H, m), 4.58 (2H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.73, 142.10 (2C), 137.14 (2C), 128.93 (2C), 127.95, 127.00 (2C), 124.37, 113.31 (2C), 46.96. HRMS (ESI^+) *m/z* calcd. for $C_{14}H_{12}N_3Cl$ $[M + H]^+$, 292.0403, found, 292.0407.

5,6-Dichloro-N-*(4-methoxybenzyl)-1*H-*benzimidazol-2-amine,* **29**

A light brown, yield: 18%, mp: 209–210 °C (methanol). IR (KBr): ν_{max} 3339, 2929, 1620, 1597, 1569, 1514, 1455, 1248, 1174, 1030, 824, 865 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 7.28 (2H, s, H-4 + 7), 7.25 (2H, d, J = 8.8 Hz, H-2′ + 6′), 6.88 (2H, d, J = 8.8 Hz, H-3′ + 5′), 4.47 (2H, s, CH_2), 3.76 (3H, s, OCH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 160.49 (C-4′), 158.30 (C-2), 139.08 (C-3a), 134.63 (C-7a), 131.98 (C-5 + 6), 129.64 (C-2′ + 6′), 124.23 (C-1′), 114.91 (C-3′ + 5′), 113.76 (C-4 + 7), 55.63 (OCH_3), 46.80 (CH_2). HRMS (ESI^+) m/z calcd. for $C_{15}H_{14}N_3OCl_2$ $[M + H]^+$, 322.0508, found, 322.0503.

*5,6-Dichloro-*N*-(3,4,5-trimethoxybenzyl)-1*H*-benzimidazol-2-amine,* **30**

A dark orange solid, yield: 20%, mp: 111–112 °C ($CHCl_3$/methanol). ^1H NMR (400 MHz, $CDCl_3$): δ 7.21 (2H, s), 6.39 (2H, s), 4.37 (2H, s), 3.76 (3H, s), 3.66 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.13, 153.21 (2C), 139.05 (2C), 137.24, 135.62 (2C), 131.42, 113.22 (2C), 105.19 (2C), 60.76, 56.37 (2C), 47.28. HRMS (ESI^+) m/z calcd. for $C_{17}H_{18}N_3O_3Cl_2$ $[M + H]^+$, 382.0720, found, 382.0717.

4.1.2.9. General procedure for the preparation of amides of types III and IV, BZs 31 to 55. The procedure applied was similar to that indicated in Section 4.1.2.5. To a solution of the corresponding acid (0.31 mmol) in dry DMF (1 mL), 1,1-carbonyldiimidazole (0.31 mmol) was added, and the mixture maintained at room temperature for 1 h. Then, the corresponding 2-aminobenzimidazole intermediate (0.22 mmol) was added and the mixture maintained at room temperature with magnetic stirring for 16 h. The progress of the reaction was monitored by TLC using ethyl acetate as eluent. After the reaction completion, the solvent was removed in vacuum and the crude product chromatographed on silica gel using ethyl acetate as eluent. BZ yields ranged within 20–73%.

N-*(5-Methyl-1*H*-benzimidazol-2-yl)benzamide* **31**

A light yellow solid, yield: 20%, mp: 244–245 °C ($CHCl_3$/methanol). IR (KBr) ν_{max}: 3345, 2950, 1683, 1645, 1600, 1470, 1190, 887, 780, 745, 683 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 8.07 (2H, dd, J_1 = 8.0; J_2 = 1.6 Hz, H-2′ + 6′), 7.62 (1H, dd, J_1 = 7.8; J_2 = 1.6 Hz, H-4′), 7.54 (2H, dd, J_1 = 8.0; J_2 = 7.8 Hz, H-3' + 5́), 7.36 (1H, d, J = 8.4 Hz, H-7), 7.28 (1H, d, J = 1.2 Hz, H-4), 7.03 (1H, dd, J_1 = 8.4; J_2 = 1.2 Hz, H-6), 2.61 (3H, s, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.36 (NHC̲O), 148.59 (C-2), 134.00 (C-3a), 133.70 (C-5), 132.45 (C-4′), 132.00 (C-7a + 1′), 128.86 (C-3′ + 5′), 128.56 (C-2′ + 6′), 123.54 (C-6), 112.30 (C-4), 112.10 (C-7), 21.47 (CH_3). HRMS (ESI^+) m/z calcd. for $C_{15}H_{14}N_3O$ $[M + H]^+$, 252.1128, found, 252.1128.

*4-Methoxy-*N*-(5-methyl-1*H*-benzimidazol-2-yl)benzamide* **32**

A white solid, yield: 20%, mp: 234–235 °C (methanol). ^1H NMR (400 MHz, CD_3OD): δ 8.04 (2H, d, J = 8.4 Hz), 7.34 (1H, d, J = 8.0 Hz), 7.29 (1H, d, J = 1.6 Hz), 7.05 (2H, d, J = 8.4 Hz), 7.02 (1H, dd, J_1 = 8.0; J_2 = 1.6 Hz), 3.88 (3H, s), 2.44 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 166.38, 162.21, 148.30, 134.20, 132.00, 130.52 (3C), 130.46, 123.94, 115.22, 114.18 (3C), 55.49, 21.49. HRMS (ESI^+) m/z calcd. for $C_{16}H_{16}N_3O_2$ $[M + H]^+$, 282.1237, found, 282.1234.

*2-Chloro-*N*-(5-methyl-1*H*-benzimidazol-2-yl)-5-nitrobenzamide* **33**

A yellow solid, yield: 41%, mp: 240–241 °C (methanol). IR (KBr): ν_{max} 3390, 3313, 2924, 1682, 1626, 1558, 1536, 1384, 1270, 1100, 1023, 800 cm^{-1}. ^1H NMR (400 MHz, C_2D_6SO): δ 12.80 (2H, brs, NH+NHC̲O), 8.58 (1H, d, J = 2.4 Hz, H-6′), 8.25 (1H, dd, J_1 = 8.8; J_2 = 2.4 Hz, H-4′), 7.78 (1H, d, J = 8.8 Hz, H-3′), 7.29 (1H, d, J = 8.4 Hz, H-7), 7.21 (1H, brs, H-4), 6.98 (1H, brd, J = 8.4 Hz, H-6′), 2.35 (3H, s, CH_3). ^{13}C NMR (100 MHz, C_2D_6SO): δ 162.30 (NHC̲O), 147.50 (C-2), 143.80 (C-5′), 140.22 (C-2′), 136.10 (C-7a), 138.20 (C-3a), 134.39 (C-5), 134.29 (C-1′), 131.43 (C-4′), 130.53 (C-3′), 126.61 (C-6′), 125.42 (C-6), 113.52 (C-4), 113.50 (C-7), 21.62 (CH_3). HRMS (ESI^+) m/z calcd. for $C_{15}H_{12}N_4O_3Cl$ $[M + H]^+$, 331.0592, found, 331.0597.

*3,5-Dimethoxy-*N*-(5-methyl-1*H*-benzimidazol-2-yl)benzamide* **34**

A light orange, yield: 73%, mp: 155–156 °C (methanol). IR (KBr): ν_{max} 3379, 2920, 1681, 1635, 1568, 1466, 1427, 1351, 1207, 1157, 1063, 832, 803 cm^{-1}. ^1H NMR (400 MHz, C_2D_6SO): δ 8.30 (1H, brs, NH + NHC̲O), 7.30 (1H, d, J = 7.2 Hz, H-7), 7.29 (2H, t, J = 2.2 Hz, H-2′ + 6′), 7.23 (1H, brs, H-4), 6.69 (1H, t, J = 2.2 Hz, H-4′), 6.94 (1H, brd, J = 7.2 Hz, H-6), 3.80 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 2.37 (3H, s, CH_3). ^{13}C NMR (100 MHz, C_2D_6SO): δ 166.99 (NHC̲O), 160.38 (C-3′), 160.30 (C-5′), 149.50 (C-2), 137.80 (C-3a), 137.10 (C-7a), 132.89 (C-5), 130.71 (C-1′), 122.80 (C-6), 113.03 (C-4), 112.99 (C-7), 106.84 (C-4′), 106.02 (C-2′ + 6′), 55.44 ((OCH_3)$_2$), 21.26 (CH_3). HRMS (ESI^+) m/z calcd. for $C_{17}H_{18}N_3O_3$ $[M + H]^+$, 312.1343, found, 312.1338.

*3,4,5-Trimethoxy-*N*-(5-methyl-1*H*-benzimidazol-2-yl)benzamide* **35**

A light brown solid, yield: 51%, mp: 139–140 °C (methanol). ^1H NMR (400 MHz, C_2D_6SO): δ 12.15 (2H, brs), 7.47 (2H, s), 7.31 (1H, d, J = 8.4 Hz), 7.24 (1H, d, J = 1.6 Hz), 6.93 (1H, dd, J_1 = 8.4; J_2 = 1.6 Hz), 3.85 (6H, s), 3.72 (3H, s), 2.36 (2H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 169.36, 153.13 (2C), 149.16, 141.80, 134.30, 132.40, 132.10, 128.81, 123.87, 112.80, 112.10, 105.86 (2C), 60.90, 55.83 (2C), 21.56. HRMS (ESI^+) m/z calcd. for $C_{18}H_{20}N_3O_4$ $[M + H]$+, 342.1148, found, 342.1445.

N-*(5-Methyl-1*H*-benzimidazol-2-yl)picolinamide* **36**

A light yellow solid, yield: 31%, mp: 206–207 °C (methanol). IR (KBr): ν_{max} 3235, 2920, 1685, 1621, 1569, 1442, 1410, 1271, 1226, 1170, 1112, 1015, 992, 899, 870, 808, 740, 695 cm^{-1}. ^1H NMR (400 MHz, C_2D_6SO): δ 8.75 (1H, ddd, J_1 = 5.5; J_2 = 1.6; J_3 = 0.9 Hz), 8.21 (1H, ddd, J_1 = 7.8; J_2 = 1.2; J_3 = 0.9 Hz), 8.10 (1H, dt, J_1 = 7.8; J_2 = 1.6 Hz), 7.72 (1H, ddd, J_1 = 7.8; J_2 = 5.5; J_2 = 1.2 Hz), 7.36 (1H, d, J = 8.1 Hz), 7.29 (1H, d, J = 1.6 Hz), 6.94 (1H, d, J_1 = 8.1; J_2 = 1.6 Hz), 2.38 (3H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 163.22 (NHC̲O), 148.93 (C-6́), 148.48 (C-2́), 145.44 (C-2), 138.28 (C-3a + 4́), 137.62 (C-7a), 131.48 (C-5), 127.61 (C-5́), 122.69 (C-6 + 3́), 115.20 (C-7), 114.13 (C-4), 21.31 (CH_3). HRMS (ESI^+) m/z calcd. for $C_{14}H_{13}N_4O$ $[M + H]^+$, 253.1084, found, 253.1074.

N-*(5-Chloro-1*H*-benzimidazol-2-yl)benzamide* **37**

A yellow solid, yield: 60%, mp: 244–245 °C (methanol). IR (KBr): ν_{max} 3256, 2917, 1685, 1622, 1563, 1440, 1410, 1271, 1225, 1172, 1113, 1015, 889, 882, 829, 749, 695, 619 cm^{-1}. ^1H NMR (400 MHz, C_2D_6SO): δ 12.29 (2H, brs, NH + NHCO), 8.10 (2H, d, J_1 = 8.0 Hz; J_2 = 1.6 Hz, H-2′ + 6′), 7.63 (1H, dd, J_1 = 7.2; J_2 = 1.6 Hz, H-4′), 7.53 (2H, dd, J = 8.0; J = 7.2 Hz, H-3′ + 5′), 7.49 (1H, d, J = 2.0 Hz, H-4), 7.45 (1H, d, J = 8.0 Hz, H-7), 7.13 (1H, dd, J_1 = 8.0; J_2 = 2.0 Hz, H-6). ^{13}C NMR (100 MHz, C_2D_6SO): δ 167.50 (NHC̲O), 149.11 (C-2), 137.60 (C-3a), 134.60 (C-7a), 133.63 (C-1′), 132.90 (C-4′), 128.94 (C-3′ + 5′), 128.68 (C-2′ + 6′), 126.01 (C-5), 121.82 (C-6), 115.17 (C-4), 114.10 (C-7). HRMS (ESI^+) m/z calcd. for $C_{14}H_{11}N_3OCl$ $[M + H]^+$, 272.0585, found, 272.0584.

N-*(5-Chloro-1*H*-benzimidazol-2-yl)-4-methoxybenzamide* **38**

A light pink solid, yield: 55%, mp: 249–250 °C (methanol). ^1H NMR (400 MHz, C_2D_6SO): δ 12.19 (2H, brs), 8.10 (2H, d, J = 8.8 Hz), 7.48 (1H, d, J = 2.0 Hz), 7.44 (1H, d, J = 8.8 Hz), 7.11 (1H, dd, J_1 = 8.8; J_2 = 2.0 Hz), 7.05 (2H, d, J = 8.8 Hz), 3.82 (3H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 166.47, 166.13, 149.11, 138.10, 136.30, 130.66 (2C), 125.81, 125.43, 121.62, 114.25 (2C), 108.10, 106.90, 55.95. HRMS (ESI^+) m/z calcd. for $C_{15}H_{13}N_3O_2Cl$ $[M + H]^+$, 302.0691, found, 302.0691.

N-*(5-Chloro-1*H*-benzimidazol-2-yl)picolinamide* **39**

A white solid, yield: 65%, mp: 223–224 °C (methanol). IR (KBr): ν_{max} 3241, 2915, 1684, 1625, 1560, 1440, 1412, 1273, 1226, 1172, 1110, 1017, 996, 899, 875, 808, 740, 695, 619 cm^{-1}. ^1H NMR (400 MHz, C_2D_6SO): δ 12.00 (2H, brs), 8.65 (1H, brd, J = 4.8 Hz, H-6′), 8.19 (1H, d, J = 7.6 Hz, H-3′), 8.60 (1H, ddd, J_1 = 8.0; J_2 =7.6; J_3 = 1.2 Hz, H-4′), 7.66 (1H, dd, J_1 = 8.0; J_2 = 4.8 Hz, H-5′), 7.50 (1H, d, J = 1.2 Hz, H-4), 7.48 (1H, d, J = 8.4 Hz, H-7), 7.12 (1H, J_1 = 8.4; J_2 = 1.2 Hz, H-6). ^{13}C NMR (100 MHz, C_2D_6SO):

δ 163.75 (NHCO), 149.30 (C-6′), 148.57 (C-2′), 147.19 (C-2), 138.70 (C-3a + 4′), 136.90 (C-7a), 128.16 (C-5′), 126.09 (C-5), 123.24 (C-3′), 121.89 (C-6), 116.10 (C-4), 115.90 (C-7). HRMS (ESI$^+$) *m/z* calcd. for $C_{13}H_{10}N_4OCl$ [M + H]$^+$, 273.0538, found, 273.0544.

N-*(5-Methoxy-1*H*-benzimidazol-2-yl)benzamide* **40**

A white solid, yield: 60%, mp: 195–196 °C ($CHCl_3$/methanol). IR (KBr): ν_{max} 3255, 2915, 1680, 1621, 1565, 1439, 1410, 1270, 1223, 1170, 1112, 1015, 889, 882, 829, 749, 695 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.83 (2H, brs), 8.13 (2H, dd, J_1 = 8.4; J_2 = 1.6 Hz, H-2′ + 6′), 7.52 (1H, m, H-4′), 7.42 (2H, dd, J_1 =8.4; J_2 = 7.2 Hz, H-3′ + 5′), 7.10 (1H, brs, H-4), 6.73 (1H, dd, J_1 = 8.4; J_2 = 2.0 Hz, H-6), 6.70 (1H, d, J = 8.4 Hz, H-7), 3.67 (3H, s, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.10 (NHCO), 156.40 (C-5), 144.20 (C-2), 136.20 (C-3a), 132.88 (C-4′), 131.88 (C-1′), 130.40 (C-7a), 128.85 (C-3′ + 5′), 128.46 (C-2′ + 6′), 111.73 (C-6), 109.70 (C-7), 108.10 (C-4), 55.75 (OCH_3). HRMS (ESI$^+$) *m/z* calcd. for $C_{15}H_{14}N_3O_2$ [M + H]$^+$, 268.1081, found, 268.1075.

4-Methoxy-N-*(5-methoxy-1*H*-benzimidazol-2-yl)benzamide* **41**

A white solid, yield: 57%, mp: 192–193 °C ($CHCl_3$/methanol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (2H, d, J = 8.4 Hz), 7.21 (1H, brs), 6.96 (2H, J = 8.4 Hz), 6.81 (1H, dd, J_1 = 9.2; J_2 = 2.4 Hz), 6.76 (1H, brs), 3.86 (3H, s), 3.83 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.56, 163.29, 156.17, 148.20, 136.40, 130.58 (2C), 130.00, 125.08, 114.07 (2C), 111.34, 107.60, 107.21, 55.64, 55.49. HRMS (ESI$^+$) *m/z* calcd. for $C_{16}H_{16}N_3O_3$ [M + H]$^+$, 298.1186, found, 298.1181.

N-*(5-Methoxy-1*H*-benzimidazol-2-yl)picolinamide* **42**

A light yellow solid, yield: 40%, mp: 175–176 °C ($CHCl_3$/methanol). IR (KBr): ν_{max} 3232, 2915, 1681, 1620, 1567, 1442, 1412, 1270, 1225, 1173, 1112, 1015, 992, 899, 870, 808, 740, 695 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.57 (1H, ddd, J_1 = 5.5; J_2 = 1.9; J_3 = 0.9 Hz, H-6′), 8.26 (1H, ddd, J_1 = 7.6; J_2 = 1.6; J_3 = 0.9 Hz, H-3′), 7.89 (1H, ddd, J_1 = 7.6; J_2 = 7.5; J_3 = 1.9 Hz, H-4′), 7.50 (1H, ddd, J_1 = 7.5; J_2 = 5.5; J_3 = 1.6 Hz, H-5′), 7.40 (1H, d, J = 8.0 Hz, H-7), 7.02 (1H, brs, H-4), 6.86 (1H, d, J = 8.0 Hz, H-6), 3.82 (3H, s, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.54 (NHCO), 156.15 (C-5), 148.65 (C-6′), 148.17 (C-2′), 147.87 (C-2), 137.02 (C-4′), 136.97 (C-3a), 129.93 (C-7a), 127.32 (C-5′), 122.73 (C-3′), 111.18 (C-6), 100.80 (C-7), 108.50 (C-4), 55.79 (OCH_3). HRMS (ESI$^+$) *m/z* calcd. for $C_{14}H_{13}N_4O_2$ [M + H]$^+$, 269.1033, found, 269.1022.

N-*(5,6-Dimethyl-1*H*-benzimidazol-2-yl)benzamide* **43**

A white solid, yield: 32%, mp: 232–233 °C (methanol). ^{1}H NMR (400 MHz, C_2D_6SO): δ 11.89 (2H, brs), 7.62 (1H, dd, J_1 = 7.8; J_2 = 1.6 Hz), 7.39 (2H, dd, J_1 = 8.0; J_2 = 7.8 Hz), 7.35 (2H, dd, J_1 = 8.0; J_2 = 1.6 Hz), 7.01 (2H, s), 2.31 (6H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 168.21, 146.13, 137.22 (2C), 132.80, 132.11, 131,74 (2C), 128.65 (2C), 128.22 (2C), 115.63 (2C), 19.16 (2C). HRMS (ESI$^+$) *m/z* calcd. for $C_{16}H_{16}N_3O$ [M + H]$^+$, 266.1293, found, 266.1299.

N-*(5,6-Dimethyl-1*H*-benzimidazol-2-yl)-4-methoxybenzamide* **44**

A white solid, yield: 28%, mp: 259–260 °C ($CHCl_3$/methanol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (2H, d, J = 8.8 Hz), 6.83 (2H, d, J = 8.8 Hz), 6.85 (2H, brs), 3.76 (3H, s), 2.16 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.06, 162.97, 148.81, 135.80 (2C), 130.61 (2C), 130.52 (2C), 126.43, 115.20 (2C), 114.03 (2C), 55.41, 20.05 (2C). HRMS (ESI$^+$) *m/z* calcd. for $C_{17}H_{18}N_3O_2$ [M + H]$^+$, 296.1394, found, 296.1392.

N-*(5,6-Dichloro-1*H*-benzimidazol-2-yl)benzamide* **45**

A white solid, yield: 26%, mp: 335–336 °C (methanol). ^{1}H NMR (400 MHz, C_2D_6SO): δ 8.09 (2H, dd, J_1 = 7.2; J_2 = 1.6 Hz), 7.69 (2H, s), 7.62 (1H, dd, J_1 = 7.6; J_2 = 1.6 Hz), 7.53 (2H, dd, J_1 = 7.6; J_2 = 7.2 Hz). ^{13}C NMR (100 MHz, C_2D_6SO): δ 166.44, 149.21, 138.10 (2C), 132.69, 128.57 (2C), 128.26 (2C), 125,90 (2C), 123.39, 115.50 (2C). HRMS (ESI$^+$) *m/z* calcd. for $C_{14}H_{10}N_3OCl_2$ [M + H]$^+$, 306.0195, found, 306.0195.

N-*(5,6-Dichloro-1*H*-benzimidazol-2-yl)-4-methoxybenzamide* **46**

A white solid, yield: 30%, mp: 295–296 °C (methanol). ^{1}H NMR (400 MHz, C_2D_6SO): δ 12.45 (1H, brs), 12.01 (1H, brs), 8.10 (2H, d, J = 8.8 Hz), 7.67 (2H, s), 7.07 (2H, d, J = 8.8 Hz), 3.84 (3H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 165.78, 162.82, 149.21, 130.50 (2C), 130.37 (2C), 124.55 (2C), 123.26, 116.10 (2C), 113.86 (2C), 55.54. HRMS (ESI$^+$) *m/z* calcd. for $C_{15}H_{12}N_3O_2Cl_2$ [M + H]$^+$, 336.0301, found, 336.0301.

N-*(5-Methyl-1*H*-benzimidazol-2-yl)-2-(3-nitrophenyl)acetamide* **47**

A white solid, yield: 72%, mp: 233–234 °C (methanol). IR (KBr): ν_{max} 3352, 2920, 1690 (NHCO), 1648 (CN), 1605, 1524, 1482, 1350, 1192, 1034, 797, 718 cm^{-1}. ^{1}H NMR (400 MHz, C_2D_6SO): δ 12.03 (2H, brs, NH + NHCO), 8.45 (1H, brs, H-2′), 8.29 (1H, d, J = 7.9 Hz, H-4′), 7.96 (1H, d, J = 7.7 Hz, H-6′), 7.79 (1H, dd, J_1 = 7.9; J_2 =7.7 Hz, H-5′), 7.43 (1H, d, J = 8.0 Hz, H-7), 7.36 (1H, brs, H-4), 7.03 (1H, brd, J = 8.0 Hz, H-6), 4.10 (2H, s, CH_2), 2.49 (3H, s, CH_3). ^{13}C NMR (100 MHz, C_2D_6SO): δ 169.59 (NHCO), 147.69 (C-3′), 146.23 (C-2), 140.50 (C-3a), 138.90 (C-7a), 137.38 (C-1′), 136.38 (C-6′), 130.06 (C-5), 129.84 (C-5′), 124.22 (C-2′), 122.40 (C-6), 121.91 (C-4′), 117.21 (C-4), 111.21 (C-7), 41.47 (CH_2), 21.34 (CH_3). HRMS (ESI$^+$) *m/z* calcd. for $C_{16}H_{15}N_4O_3$ [M + H]$^+$, 311.1139, found, 311.1134.

2-Hydroxy-N-*(5-methyl-1*H*-benzimidazol-2-yl)-2-phenylacetamide* **48**

A light yellow solid, yield: 17%, mp: 243–244 °C ($CHCl_3$/methanol). IR (KBr): ν_{max} 3368, 3212, 2922, 1686, 1620, 1575, 1450, 1212, 1067, 810, 694 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42 (2H, d, J = 6.4 Hz, H-2′ + 6′), 7.21 (3H, m, H-3′ + 4′ + 5′), 7.14 (1H, d, J = 8.0 Hz, H-7), 7.05 (1H, s, H-4), 6.91 (1H, d, J = 8.0 Hz, H-6), 5.29 (1H, s, H-α), 2.31 (3H, s, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.32 (NHCO), 146.88 (C-2), 138.20 (C-3a), 134.50 (C-7a), 132.72 (C-5), 128.87 (C-1′), 128.71 (C-3′ + 5′), 128.65 (C-4′), 126.65 (C-2′ + 6′), 124.15 (C-6), 112.30 (C-4), 110.20 (C-7), 75.12 (CH-α), 21.53 (CH_3). HRMS (ESI$^+$) *m/z* calcd. for $C_{16}H_{16}N_3O_2$ [M + H]$^+$, 282.1237, found, 282.1242.

N-*(5-Methyl-1*H*-benzimidazol-2-yl)-2-(naphthalen-2-yl)acetamide* **49**

A light yellow solid, yield: 32%, mp: 245–246 °C (methanol). IR (KBr): ν_{max} 3359, 2924, 1680 (NHCO), 1645, 1598, 1482, 1433, 1220, 810, 794, 739, 670 cm^{-1}. ^{1}H NMR (400 MHz, C_2D_6SO): δ 11.90 (1H, brs, NH), 11.78 (1H, brs, NHCO), 7.92 (1H, brd, J = 8.8 Hz, H-5′), 7.88 (1H, brd, J = 8.6 Hz, H-8′), 7.81 (1H, d, J = 7.6 Hz, H-4′), 7.69 (1H, d, J = 1.6 Hz, H-1′), 7.46 (1H, m, H-6′), 7.44 (1H, m, H-7′), 7.39 (1H, dd, J_1 = 7.6; J_2 = 1.6 Hz, H-3′), 7.32 (1H, d, J = 8.4 Hz, H-7), 7.25 (1H, d, J = 1.6 Hz, H-4), 6.89 (1H, dd, J_1 = 8.4; J_2 = 1.6 Hz, H-6), 3.92 (2H, s, CH_2), 2.33 (3H, s, CH_3). ^{13}C NMR (100 MHz, C_2D_6SO): δ 171.99 (NHCO), 146.18 (C-2), 141.20 (C-3a), 140.39 (C-3′), 138.60 (C-7a), 134.91 (C-2′), 133.47 (C-8′a), 132.96 (C-5), 132.66 (C-4′a), 128.73 (C-5′), 127.74 (C-8′), 127.62 (C-4′), 126.40 (C-1′), 126.16 (C-7′), 125.03 (C-6′), 117.10 (C-6), 111.30 (C-4), 111.10 (C-7), 42.42 (CH_2), 21.35 (CH_3). HRMS (ESI$^+$) *m/z* calcd. for $C_{20}H_{18}N_3O$ [M + H]$^+$, 316.1444, found, 316.1440.

N-*(5-Methyl-1*H*-benzimidazol-2-yl)thiophene-2-acetamide* **50**

A white solid, yield: 47%, mp: 230–231 °C ($CHCl_3$/methanol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33 (1H, d, J = 4.8 Hz), 7.32 (1H, d, J = 8.0 Hz), 7.24 (1H, d, J = 1.2 Hz), 7.15 (1H, d, J_1 = 6.5; J_2 = 4.8 Hz), 7.06 (1H, d, J = 6.5 Hz), 7.04 (1H, dd, J_1 = 8.0; J_2 = 1.2 Hz), 3.94 (2H, s), 2.44 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.36, 147.58, 138.10, 133.12, 132.43, 130.10, 128.25, 126.42, 123.45, 110.00, 108.10, 107.10, 38.15, 21.62. HRMS (ESI$^+$) *m/z* calcd. for $C_{14}H_{14}N_3OS$ [M + H]$^+$, 272.0852, found, 272.0860.

3-Chlorophenyl-N-*(5,6-dimethyl-1*H*-benzimidazol-2-yl)acetamide* **51**

A light brown solid, yield: 42%, mp: 264–265 °C (methanol). ^{1}H NMR (400 MHz, C_2D_6SO): δ 11.78 (2H, brs), 7.43 (1H, brs), 7.35 (1H, dd, J_1 = 8.0; J_2 = 7.8 Hz), 7.32 (1H, dd, J_1 = 8.0; J_2 = 1.6 Hz), 7.29 (1H, dd, J_1 = 7.8; J_2 = 1.6 Hz), 7.17 (2H, s), 3.75 (2H, s), 2.23 (6H, s). ^{13}C NMR (100 MHz, C_2D_6SO): δ 169.72, 145.77, 138.10 (2C),

137.66, 132.85, 130.17, 129.18, 129.10 (2C), 128.02, 126.75, 111.83 (2C), 41.72, 19.91 (2C). HRMS (ESI$^+$) *m/z* calcd. for $C_{17}H_{17}N_3OCl$ $[M + H]^+$, 314.1055, found, 314.1045.

N-*(5,6-Dichloro-1*H*-benzimidazol-2-yl)-2-(3-nitrophenyl)acetamide* **52**

A orange solid, yield: 68%, mp: 287–289 °C (methanol). IR (KBr): ν_{max} 3327, 2924, 1690, 1637, 1581, 1526, 1453, 1349, 1297, 1124, 1040, 854, 810, 790, 672, 659 cm^{-1}. ^{1}H NMR (400 MHz, C_2D_6SO): δ 12.31 (1H, brs, NH), 12.05 (1H, brs, NHCO), 8.13 (1H, dd, J_1 = 7.6; J_2 = 2.0; J_3 = 1.8 Hz, H-4′), 7.79 (1H, dd, J_1 = 8.0; J_2 = 7.6 Hz, H-5′), 7.65 (1H, ddd, J_1 = 8.0; J_2 = 2.2; J_3 = 2.0 Hz, H-6′), 7.61 (1H, dd, J_1 = 2.2; J_2 = 1.8 Hz, H-2′), 7.56 (2H, s, 4 + H-7), 3.98 (2H, s, CH_2). ^{13}C NMR (100 MHz, C_2D_6SO): δ 170.30 (NHCO), 148.77 (C-3′), 148.12 (C-2), 140.95 (C-3a + 7a), 136.77 (C-6′), 132.74 (C-1′), 130.21 (C-5′), 124.68 (C-2′), 123.97 (C-6), 123.21 (C-5), 122.34 (C-4′), 118.58 (C-4), 113.12 (C-7), 41.78 (CH_2). HRMS (ESI$^+$) *m/z* calcd. for $C_{15}H_{10}N_4O_3Cl_2$ $[M + H]^+$, 365.0208, found, 365.0015.

Tert-*butyl(1-((5-methoxy-1*H*-benzimidazol-2-yl)amino)-1-oxododecan-2-yl-carbamate* **54**

A yellow solid, yield: 62%, mp: 113–114 °C ($CHCl_3$/methanol). IR (KBr): ν_{max} 3347, 2919, 2850, 1683, 1642, 1596, 1483, 1462, 1229, 1200, 1029, 857, 787 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.12 (1H, brs, H-4), 6.86 (1H, brd, J = 8.0 Hz, H-6), 6.11 (1H, d, J = 8.0 Hz, H-7), 4.68 (1H, m, H-α), 3.82 (3H, s, OCH_3), 1.75 (2H, m), 1.74 (2H, m), 1.45 (9H, s, $(CH_3)_3$), 1.23 (14H, m), 0.84 (3H, t, J = 6.8 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.13 (CONH), 155.68 (5-C), 156.09 (OCOC$(CH_3)_3$), 146.87 (C-2), 133.21 (C-3a), 130.00 (C-7a), 111.40 (C-6), 111.27 (C-7), 95.64 (C-4), 79.64 (OCOC$(CH_3)_3$), 55.65 (OCH_3), 54.99 (CH- α), 31.95, 31.78, 29.61, 29.46, 29.38, 29.33, 29.20, 28.25 (OCOC$(CH_3)_3$), 22.58, 22.40, 14.03 (CH_3). HRMS (ESI$^+$) *m/z* calcd. for $C_{25}H_{41}N_4O_4$ $[M + H]^+$, 461.3122, found, 461.3122.

4.1.2.10. Obtaining BZ 53 by reduction of the nitrobenzimidazole 52. The same protocol than that indicated in 4.1.2.2 was applied. To a solution of BZ **52** (40 mg, 0.109 mmol) in MeOH (3 mL) placed in a two-necked round bottom flask, Pd-C catalyst (3 mg) was added. The air was removed, and a balloon with H_2 was placed to one of the necks. The mixture was kept at room temperature and under magnetic stirring for 2 h. Then, the Pd-C was removed by filtration through Celite, and the residue was washed with MeOH. The solvent was removed in vacuum in a rotary evaporator to provide a solid that was purified by crystallisation in methanol, 14 mg, 39% yield.

*2-(3-Aminophenyl)-*N*-(5,6-dichloro-1*H*-benzimidazol-2-yl)acetamide* **53**

A light yellow solid, mp: 298–299 °C. IR (KBr): ν_{max} 3420, 3327, 2924, 1695, 1637, 1581, 1526, 1408, 1349, 1297, 1124, 854, 790, 730 cm^{-1}. ^{1}H NMR (400 MHz, CD $_3$OD): δ 7.59 (2H, s, H-4 + 7), 7.06 (1H, dd, J_1 = 8.0; J_2 = 7.6 Hz, H-5′), 6.72 (1H, brs, H-2′), 6.69 (1H, d, J = 7.6 Hz, H-6′), 6.63 (1H, brd, J = 8.0 Hz, H-4′), 3.68 (2H, s, CH_2). ^{13}C NMR (100 MHz, CD $_3$OD): δ 173.06 (NHCO), 150.01 (C-3′), 149.17 (C-2), 141.20 (C-3a + 7a), 136.38 (C-1′), 130.40 (C-5′), 126.80 (C-5 + 6), 119.91 (C-6′), 117.16 (C-2′), 115.45 (C-4′), 113.76 (C-4 + 7), 43.97 (CH_2). HRMS (ESI$^+$) *m/z* calcd. for $C_{15}H_{13}N_4OCl$ $[M + H]^+$, 335.0466, found, 335.0457.

4.1.2.11. Obtaining BZ 55 by deprotection of the Boc group in 54. 100 mg (0.217 mmol) of compound **54** were dissolved in CH_2Cl_2 (3 mL) and kept at 0 °C under stirring. Then, trifluoroacetic acid (1.0 mL) was added. The reaction was monitored every 15 min. by TLC *n*-hexane/ethyl acetate (4:6) using as eluent. After completion of the reaction, it was extracted with CH_2Cl_2, washed with water and the organic layer over anhydrous sodium sulphate. The solvent was removed under vacuo to provide a crude mixture that was purified by column chromatography with *n*-hexane/ethyl acetate (4:6) as eluent to provide BZ **55** 27 mg in 35% yield.

N-*(5-methoxy)-1*H*-benzimidazol-2-yl)-2-aminododecanamide* **55**

A white solid, yield: 27%, mp: 105–106 °C ($CHCl_3$/methanol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42 (1H, brs), 7.13 (1H, d, J = 8.8 Hz), 6.85 (1H, brd, J = 8.8 Hz), 5.92 (1H, d, J = 9.2 Hz, NHCO), 4.69 (1H, m, CH- α), 4.62 (2H, brs, NH_2), 3.82 (3H, s), 1.79 (2H, m), 1.65 (2H, m), 1.42 (2H, m), 1.21 (6H, m), 0.84 (3H, t, J = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.54, 157.17, 143.70, 133.07, 129.60, 112.63, 111.99, 96.30, 61.83, 56.19, 35.06, 32.90. 30.72, 30.46, 30.37, 30.35, 30.31, 26.73, 23.68, 15.12. HRMS (ESI$^+$) *m/z* calcd. for $C_{20}H_{33}N_4O_2$ $[M + H]^+$, 361.2598, found, 361.2603.

4.1.3. Molecular docking

T. circumcinta β-tubulin obtained by homology modelling used as a target for docking studies was the same as has been described in a previous work.[8] Docking calculations were performed using GLIDE integrated into the Schrödinger Molecular Modelling Suite (Schrödinger, Inc., USA, 2016.4) according the methodology reported in the work; with the only difference of allowing the free rotation of the hydroxyl and sulfanyl groups of the colchicine site during the grid generation.

4.2. Biological assays

More than fifty BZs derivatives were screened *in vitro* to prove their effects on eggs and third-stage larvae (L3) of *T. circumcincta* by means of the Egg Hatch Assay (EHA) and the Larval Migration Inhibition Assay (LMIA), respectively. Cytotoxicity assays were also performed on Caco-2 and HepG2 cells in order to calculate the selectivity index (SI) of each compound.

4.2.1. Animals

Two animals were used to obtain the necessary material to carry out the *in vitro* experiments. Two months-old Merino lambs were infected with 20,000 L3 of *T. circumcincta.* All protocols carried out were performed according to current national and European regulations of animal wellbeing (R.D 53/2013 and EU Directive 2010/63/EU) at the facilities of the Instituto de Ganadería de Montaña (IGM, CSIC, León, Spain).

4.2.2. Egg hatch assay (EHA)

The eggs were extracted from fresh faecal material of infected animals by sieving, centrifugation and flotation in a solution of saturated sodium-chloride. Each compound was first tested in a susceptible strain of the GIN at concentration of 50 μM in order to select those with activities higher than 97%. Then, their half maximal effective concentration (EC_{50}) was calculated using eight serial dilutions (1:2) ranging from 50 to 0.39 μM. Thiabendazole at 0.49 μM (equivalent to 0.1 μg/mL; the known cut-off value used to classify field susceptible and resistant strains according to Coles et al. [36] was used as positive control and DMSO at 0.5% as negative. The EHA was performed using a similar protocol described by Coles et al. [36] Briefly, each compound was incubated with fresh eggs in 24-well culture plates for 48 h at 23 °C. The concentration of eggs per well was 150 in a final volume of 2 mL. All compounds were tested in duplicate during three different days to ensure the accuracy of results. After 48 h of incubation, all eggs and larvae present in each well were counted and the ovicidal activity was estimated by following the formulas:

% Egg hatching per well

$$= (\text{number of L1}/\text{number of L1 larvae and eggs}) \times 100.$$

% Ovicidal activity

$$= [100 - (\%\ \text{Egg hatching per well}/\%\ \text{Egg hatching in control well})] \times 100.$$

Dose-response curves were fitted by nonlinear regression using the computer program Sigma Plot V 10.0 (Systat Software, Inc., San José, California; USA). The EC_{50} values and R^2 values were calculated. The results were expressed as the mean of the EC_{50} and the standard error of the mean (SEM).

4.2.3. Larval migration inhibition assay

Infective L3 obtained from coprocultures were used to carry out the LMIA. For this, fresh faeces from infected lambs were cultured in small closed boxes aerated and humidified twice a week and placed in a climatic chamber at 25 °C for ten days to allow the hatching of eggs and the development of the larvae to the infective stage. Then, the larvae were stored in ventilated cell culture flasks at 6–10 °C in a fridge for a maximum period of three months.

The assay was performed similarly to the method described previously by Demeler et al.[37] and the procedure followed was the same as for the EHA. Firstly, an initial screening of all compounds at 50 µM was performed. Briefly, L3 were incubated with the different compounds for 24 hours in the dark at 28 °C in 48-well culture plates. Then, the content of the wells was transferred to a 96-wells MultiScreen-Mesh Filter Plate (Sigma-Aldrich) and left for a further 24 h period to allow the motile L3 to pass through the sieves for counting. 0.5% DMSO was used as negative control in each assay.

The efficacy of the compound was expressed as the larval migration inhibition following the formula:

% Larval migration inhibition = [(number of larvae migrating through sieves in negative controls wells – number of larvae migrating through sieves in treatment wells) / number of larvae migrating through sieves in negative controls wells.] x 100.

4.3. Cytotoxicity assay

Those compounds with activities higher than 95% at a 50 µM concentration in the initial screening were selected to carry out cytotoxicity assays on two human cell lines: the human colorectal adenocarcinoma Caco-2 (ATCC® HTB-37™) and the human hepatocarcinoma HepG2 (ATCC® HB-8065™), to assess the toxicity on a cell line of intestinal origin and to assess the systemic toxicity of the compounds, respectively.

Briefly, 10,000 cells were seeded on 96 well-plates containing RPMI 1640 Medium supplemented with 2.0 g/L sodium bicarbonate (Fisher Scientific®), 1% (w/v) L-glutamine (Sigma-Aldrich®) and 25 mM HEPES buffer, pH 7.6, 10% (v/v) inactivated foetal bovine serum and antibiotic cocktail containing 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, different concentrations of testing compounds (ranging 1 to 100 µM) were added for a period of 72 h. After this time, the viability of the cells was assessed using the Alamar Blue (Thermo Fisher) staining method according to manufacturer's recommendations.

Cell viability expressed as the fluorescence emitted by resorufin at 590 nm was plotted against the corresponding concentration added to cell culture and fitted using the software package for scientific data analysis SigmaPlot 10.0 with the aim to estimate the half cytotoxic concentration (CC_{50}) of each compound. As an estimation of the safety of each compound, selectivity index (SI) with respect to HepG2 cells was calculated by dividing CC_{50} by EC_{50}.

Supporting information

^1H, ^{13}C NMR and HRMS spectra of relevant compounds. Additional results of docking studies. Physicochemical Drug-Likeness and Toxicity risks for selected BZs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Financial support came from MINECO: RETOS (AGL2016-79813-C2-1R/2R) and Junta de Castilla y León co-financed by FEDER, UE [LE020P17]. EVG was funded by FPU17/00627; VCGA and MAB are recipients of Junta de Castilla y Leon (JCyL) (LE082-18, LE051-18, respectively) and MMV by the Spanish "Ramon y Cajal" Programme (Ministerio de Economía y competitividad; MMV, RYC-2015-18368).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2022.133735.

References

[1] R.L. Pullan, J.L. Smith, R. Jasrasaria, S.J. Brooker, Global numbers of infection and disease burden of soil transmitted helminth infections in 2010, Parasites Vectors 7 (2014) 1–19, doi:10.1186/1756-3305-7-37.
[2] T.M. Craig, Gastrointestinal nematodes, diagnosis and control, Vet. Clin. North Am. Food Anim. Pract. 34 (2018) 185–199, doi:10.1016/j.cvfa.2017.10.008.
[3] F. Jackson, R.L. Coop, The development of anthelmintic resistance in sheep nematodes, Parasitology (2000) 120, doi:10.1017/s0031182099005740.
[4] E. Papadopoulos, E. Gallidis, S. Ptochos, Anthelmintic resistance in sheep in Europe: a selected review, Vet. Parasitol. 189 (2012) 85–88, doi:10.1016/j.vetpar.2012.03.036.
[5] M. Martínez-Valladares, T. Geurden, D.J. Bartram, J.M. Martínez-Pérez, D. Robles-Pérez, A. Bohórquez, E. Florez, A. Meana, F.A. Rojo-Vázquez, Resistance of gastrointestinal nematodes to the most commonly used anthelmintics in sheep, cattle and horses in Spain, Vet. Parasitol. 211 (3–4) (2015) 228–233.
[6] M. Zajíčková, L.T. Nguyen, L. Skálová, L. Raisová Stuchlíková, P. Matoušková, Anthelmintics in the future: current trends in the discovery and development of new drugs against gastrointestinal nematodes, Drug Discov. Today (2020), doi:10.1016/j.drudis.2019.12.007.
[7] E.L. Gutiérrez, M.S. Souza, L.F. Diniz, J. Ellena, Synthesis, characterization and solubility of a new anthelmintic salt: mebendazole nitrate, J. Mol. Struct. 1161 (2018) 113–121, doi:10.1016/j.molstruc.2018.02.060.
[8] N. Escala, E. Valderas-García, M.Á. Bardón, V.C. Gómez de Agüero, R. Escarcena, J.L. López-Pérez, F.A. Rojo-Vázquez, A. San Feliciano, R. Balaña-Fouce, M. Martínez-Valladares, E. Olmo, Synthesis, bioevaluation and docking studies of some 2-phenyl-1*H*-benzimidazole derivatives as anthelminthic agents against the nematode *Teladorsagia circumcincta*, Eur. J. Med. Chem. 208 (2020) 112554, doi:10.1016/j.ejmech.2020.112554.
[9] M. Abongwa, R.J. Martin, A.P. Robertson, A brief review on the mode of action of antinematodal drugs, Acta Vet 67 (2017) 137e152, doi:10.1515/acve-2017-0013.
[10] Spartan20. Wavefunction, Inc. 18401 Von Karman Ave., Suite 435. Irvine, CA 92612. https://www.wavefun.com/.
[11] U.S. Sørensen, D. Strøbæk, P. Christophersen, C. Hougaard, M.L. Jensen, E.Ø. Nielsen, D. Peters, L. Teuber, Synthesis and structure−activity relationship studies of 2-(*N*-Substituted)-aminobenzimidazoles as potent negative gating modulators of small conductance Ca^{2+}-activated K^+ channels, J. Med. Chem. 51 (23) (2008) 7625–7634, doi:10.1021/jm800809f12.
[12] J. Ha Lee, M. Hyun An, E. Hyun Choi, H.Y. Park Choo, G. Han, a facile synthesis of 2-acyl and 2-alkylaminobenzimidazoles for 5-lipoxygenase Inhibitors, Heterocycles 70 (1) (2006) 571, doi:10.3987/com-06-s(w)25.
[13] T.C. Kühler, M. Swanson, B. Christenson, A.C. Klintenberg, B. Lamm, J. Fägerhag, R. Gatti, M. Ölwegard-Halvarsson, V. Shcherbuchin, T. Elebring, J.E. Sjöström, Novel structures derived from 2-[[(2-Pyridyl)methyl]thio]-1*H*-benzimidazole as anti-*Helicobacter pylori* agents, Part 1, J. Med. Chem. 45 (19) (2002) 4282–4299, doi:10.1021/jm0208673.
[14] NF. Abdel-Ghaffar, Synthesis, reactions, structure-activity relationship of 2-benzimidazole analogs as anticancer agents and study their molecular docking, Pharm. Chem. 5 (5) (2013) 243–257.
[15] W. Nawrocka, B. Sztuba, M.W. Kowalska, H. Liszkiewicz, J. Wietrzyk, A. Nasulewicz, M. Pelczyńska, A. Opolski, Synthesis and antiproliferative activity *in vitro* of 2-aminobenzimidazole derivatives, Farm. Sci. 59 (2) (2004) 83–91, doi:10.1016/j.farmac.2003.12.001.

[16] V.S. Pilyugin, Y.E. Sapozhnikov, N.A. Sapozhnikova, Acyl derivatives of 2-aminobenzimidazole and their fungicide activity, Russ. J. Gen. Chem. 74 (5) (2004) 738–743, doi:10.1023/b:rugc.0000039088.870.
[17] V.S. Pilyugin, Y.E. Sapozhnikov, A.M. Davydov, G.E. Chikisheva, T.P. Vorob'eva, E.V. Klimakova, G.V. Kiseleva, S.L. Kuznetsova, R.D. Davletov, N.A. Sapozhnikova, R.K. Yumadilov, 13C NMR spectra and biological activity of *N*-(1*H*-benzimidazol-2-yl)benzamides, Russ. J. Gen. Chem. 76 (10) (2006) 1653–1659, doi:10.1134/s1070363206100264.
[18] R.D. Davletov, G.E. Chikisheva, R.N. Galiakhmetov, Search for environmentally less harmful fungicides in a series of benzimidazole derivatives, Bašk. Him. Ž. 17 (2) (2010) 28–32.
[19] G.E. Chikisheva, Y.E. Sapozhnikov, R.K. Mudarisova, L.I. Buslaeva, Z.B. Galieva, R.D. Davletov, Synthesis and fungicidal activity of 2-benzoylaminobenzimidazole complex compounds with copper, Russ. J. Appl. Chem. 86 (2) (2013) 285–288, doi:10.1134/s10704272130226220.
[20] K.L. Obydennov, T.A. Kalinina, N.A. Galieva, T.V. Beryozkina, Y. Zhang, Z. Fan, T.V. Glukhareva, V.A. Bakulev, Synthesis, fungicidal activity and molecular docking of 2-acylamino and 2-thioacylamino derivatives of 1*H*-benzo[*d*]imidazoles as anti-tubulin agents, J. Agric. Food Chem. 69 (40) (2021) 12048–12062, doi:10.1021/acs.jafc.1c03325.
[21] F. Odame, R. Betz, E.C. Hosten, J. Krause, M. Isaacs, H.C. Hoppe, S.D. Khanye, Y. Sayed, C. Frost, K.A. Lobb, Z.R. Tshentú, A new synthetic method for tetraazatricyclic derivatives and evaluation of their biological properties, ChemistrySelect 3 (48) (2018) 13613–13618, doi:10.1002/slct.20180293022.
[22] K. Garg, Y. Bansal, G. Bansal, R.K. Goel, Design, synthesis, and PASS-assisted evaluation of novel 2-substituted benzimidazole derivatives as potent anthelmintics, Med. Chem. Res. 23 (2014) 2690–2697, doi:10.1007/s00044-013-0856-1.
[23] K. Ding, A. Wang, M.A. Boerneke, S.M. Dibrov, T. Hermann, Aryl-substituted aminobenzimidazoles targeting the hepatitis C virus internal ribosome entry site, Bioorg. Med. Chem. Lett. 24 (14) (2014) 3113–3117, doi:10.1016/j.bmcl.2014.05.009.
[24] X. He, S.K. Lakkaraju, M. Hanscom, Z. Zhao, J. Wu, B. Stoica, A.D. MacKerell, A.I. Faden, F. Xue, Acyl-2-aminobenzimidazoles: a novel class of neuroprotective agents targeting mGluR5, Bioorg. Med. Chem. 23 (9) (2015) 2211–2220, doi:10.1016/j.bmc.2015.02.05425.
[25] J.P. Powers, S. Li, J.C. Jaen, J. Liu, N.P.C. Walker, Z. Wang, H. Wesche, Discovery and initial SAR of inhibitors of interleukin-1 receptor-associated kinase-4, Bioorg. Med. Chem. Lett. 16 (11) (2006) 2842–2845, doi:10.1016/j.bmcl.2006.03.020.
[26] K. Starcevic, I. Caleta, D. Cincic, B. Kaitner, M. Kralj, K. Ester, G. Karminski-Zamola, Synthesis, crystal structure determination, and antiproliferative evaluation of novel benzazolyl benzamides, Heterocycles 68 (11) (2006) 2285–2299, doi:10.3987/COM-06-10844.
[27] E. Valderas-García, J. de la Vega, M.Á. Bardón, V.C.G. de Agüero, R. Escarcena, J.L. López-Pérez, F.A. Rojo-Vázquez, A. San Feliciano, E. del Olmo, R. Balaña-Fouce, M. Martínez-Valladares, Anthelmintic activity of aminoalcohol and diamine derivatives against the gastrointestinal nematode *Teladorsagia circumcincta*, Vet. Parasitol. 296 (2021) 109496, doi:10.1016/j.vetpar.2021.109496.
[28] Y. Bansal, M. Kaur, O. Silakari, Benzimidazole–ibuprofen/mesalamine conjugates: potential candidates for multifactorial diseases, Eur. J. Med. Chem. 89 (2015) 671–682, doi:10.1016/j.ejmech.2014.10.081.
[29] K.S. Tikhomirova, I.E. Tolpygin, A.B. Starikov, M.A. Kaz'mina, New fluorogenic chemosensors derived from benzimidazole, Chem. Heterocycl. Compd. 53 (2017) 179–185, doi:10.1007/s10593-017-2037-5.
[30] E.R. Morgan, J. van Dijk, Climate and the epidemiology of gastrointestinal nematode infections of sheep in Europe, Vet. Parasitol. 189 (2012) 8–14, doi:10.1016/J.VETPAR.2012.03.028.
[31] P.J. Dawson, W.E. Gutteridge, K. Keith Gull, A comparison of the interaction of anthelmintic benzimidazoles with tubulin isolated from mammalian tissue and the parasitic nematode *Ascaridia galli*, Biochem. Pharmacol. 33 (7) (1984) 1069–1074.
[32] M.M. Barrowman, S.E. Marriner, J.A. Bogan, The binding and subsequent inhibition of tubulin polymerization in *Ascaris suum (in vitro)* by benzimidazole anthelmintics, Biochem. Pharmacol. 33 (19) (1984) 3037–3040.
[33] Y. Zhou, J. Xu, Y. Zhu, Y. Duan, M. Zhou, Mechanism of action of the benzimidazole fungicide on *Fusarium graminearum*: interfering with polymerization of monomeric tubulin but not polymerized Microtubule, Phytopathology 106 (8) (2016) 807–813 2016 Aug, doi:10.1094/PHYTO-08-15-0186-R.
[34] J.W. Tracy, L.T. Webster, Drugs used in the chemotherapy of helminthiasis, in: J.G. Hardman, L.E. Limbind, A.G. Gilman (Eds.), Goodman and Gilman's the Pharmacological Basis of Therapeutics, l0th ed, E-McGraw-Hill, 2001, pp. 1134–1136.
[35] W.L.F. Armarego, D.D. Perrin, Purification of Laboratory Chemicals, 4th ed, Butterworth-Heinemann, 1997.
[36] G.C. Coles, F. Jackson, W.E. Pomroy, R.K. Prichard, G. Von Samson-Himmelstjerna, A. Silvestre, M.A. Taylor, J. Vercruysse, The detection of anthelmintic resistance in nematodes of veterinary importance, Vet. Parasitol. 136 (2006) 167–185, doi:10.1016/j.vetpar.2005.11.019.
[37] J. Demeler, U. Küttler, G. von Samson-Himmelstjerna, Adaptation and evaluation of three different *in vitro* tests for the detection of resistance to anthelmintics in gastrointestinal nematodes of cattle, Vet. Parasitol. 170 (2010) 61–70, doi:10.1016/J.VETPAR.2010.01.032.