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Synthesis and Anthelmintic Evaluation of Novel Valerolactam-Benzimidazole Hybrids

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Abstract: Some novel valerolactam derivatives of 5(6)-substituted-(1*H*-benzimidazol-2-yl-amine) were constructed based on the union of two structural domains with anthelmintic activity. The tested hybrid compounds 7-9 exhibited greater activity using the *Nippostrongylus brasiliensis* physiology-based *in vitro* bioassay in comparison to commercial anthelmintic benzimidazoles. Moreover, the improved physicochemical properties of hybrid compounds compared with valerolactam domain allowed the penetration of parasite barriers, concurrently with an increased intraparasitary bioavailability.

Keywords: Anthelmintic activity, N. brasiliensis, Benzimidazole carbamates, Hybrids, Valerolactam-benzimidazoles.

1. INTRODUCTION

Parasitic infections, particularly those produced by nematodes, are of interest both in human and veterinary health. The major human soil-transmitted helminths, *Ascaris lumbricoides*, *Necator americanus, Ancylostoma duodenale* and *Trichuris trichiura*, which are among the most prevalent parasites, cause more than one billion cases of infection worldwide [1]. In domestic animals, *Teladorsagia circumcincta*, *Haemonchus contortus* and *Trichostrongylus colubriformis* have evolved to become the major production-limiting nematode parasite species affecting sheep, thus causing substantial economic losses [2].

Three main types of broad-spectrum anthelmintic drugs (benzimidazoles, imidothiazoles, and macrocyclic lactones) are commercially available for the chemotherapy of nematode infections in livestock. However, despite high initial efficacy, the continued heavy use of these compounds has led many helminth strains to develop resistance. Current research is focused on the discovery of anthelmintic drugs with a novel chemical structure and acting in different biological pathways [3], even if the associated mechanisms of action are identified at a later stage [4], such as amino-acetonitrile derivatives [5] and depsipeptides [6].

The marketed anthelmintics were generally discovered by screening compounds against intact parasites in physiology-based assays, where the phenotypic parameters (viability, motility, growth, etc.) of the worms were recorded. However, mechanism-based drug screening [7] and more recently, proteomic based studies were conducted [8], but these approaches are still limited to the search for new anthelmintics, therefore, it might be convenient to combine both approaches [9].

In the search for new anthelmintic, our group developed physiology-based assays using the rat parasitic nematode *Nippostrongylus brasiliensis* L4, for preliminary *in vitro* and *in vivo* drug screening [10] and [11]. *N. brasiliensis* is closely related (analyzed by cross-taxon similarity) to the human hookworms (*Ancylostoma* and *Necator*) and to *H. contortus* (nematode of interest in sheep) [12]. These bioassays using *N. brasiliensis* were reproducible and standardized with commercially available anthelmintic, which corrrelated well with other bioassays, as we communicated previously [13].

Among the numerous compounds we have assayed [14, 15, 16] it is important to highlight a new series of 2-amine-δ-valerolactam derivatives. We reported the synthesis and anthelmintic activity of those compounds, which were extraordinary compared with albendazole and febendazole used as reference drugs [10].

It should also be noted that bioactive compounds must reach their molecular target in sufficient concentration and time to trigger the effect [17]. Hence compounds must penetrate parasite membrane barriers, physicochemical properties will be crucial for an adequate bioavailability within the parasite [18].

Nonetheless, and even when nematode *in vitro* assays are designed to mimic the physiological situation within the host, it was noted that parasites are cultured in the presence of compounds for longer (days) in comparison with the *in vivo* situation (e.g. one oral dose). Thus, a bioactive *in vitro* compound with a slow rate of intraparasitary diffusion could mask a good bioavailability into parasite.

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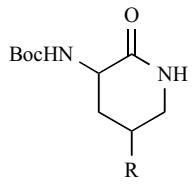


(1) R= OH
(2) R= H

Fig. (1). Chemical structures of δ-valerolactam derivatives.

This could be the case of valerolactam derivatives (Fig. **1**), which showed extraordinary *in vitro* anthelmintic activity [10] but, did not penetrate parasite barrier and showed lack of efficacy in the *in vivo* bioassay, as demonstrated herein.

In this regard, the study of the *ex vivo* ability of compounds to diffuse into the target parasite could be an interesting tool, jointly with physiology-based assays, to search for anthelmintic.

On the other hand, hybrid compounds are used in order to correct problems such as drug resistance, potency, pharmacokinetics, etc., generated by a separate component. This strategy designs new prototypes based on the union of structural domains that have different and dual activity, indicating that a hybrid molecule acts as two distinct pharmacophores [19, 20]. In this sense, the versatile core contained in benzimidazole derivatives makes it an important pharmacophore, encompassing a diverse range of biological activities, such as anthelmintic [21].

This work focuses on the synthesis of some novel valerolactam acetic acids derivatives of 5(6)-substituted-(1H-benzimidazol-2-yl-amino) based on the union of two active fragments, in order to improve phisicochemical properties with respect to valerolactam moiety.

2. EXPERIMENTAL

2.1. Chemistry

General Procedures

All reactions were carried out in dry, freshly distilled solvents under anhydrous conditions unless otherwise stated. Yields are reported for chromatographically and spectroscopically (^{1}H and ^{13}C NMR) pure compounds. Flash column chromatography was carried out with Silica gel 60 (J.T.Baker, 40 mm average particle diameter). All reactions and chromatographic separations were monitored by TLC, conducted on 0.25 mm Silica gel plastic sheets (Macherey/Nagel, Polygram, SIL G/UV254). TLC plates were analyzed under 254 nm UV light, and ninhydrine spray.

NMR spectra (^{1}H and ^{13}C) were recorded on a Bruker Avance DPX-400. Chemical shifts were related to internal standard TMS. Coupling constants (*J* values) are in Hertz (Hz). The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet, br = broad.

High resolution mass spectra (HRMS) analysis was recorded using micro Q-TOF (Bruker Daltonics) and low resolution mass spectra (LRMS) analysis was done using a Shimadzu QP 1100-EX equipment by direct injection.

FTIR spectroscopy was performed in a Fourier Transform Infrared spectrophotometer IR-Prestige 21 Shimadzu. Raman spectra of compounds 7 and 10 were done in KBr, at a concentration of 5%, in a Raman Spectrometer DeltaNu Advance. The laser was at 532 nm. Integration time of 10 seconds (20 averageable measures) and total time of measurement of 200 seconds were used. The baseline coefficient for fluorescence was 150 and Savitsky-Golay smoothing coefficient was 20. All solvents used were HPLC grade and were obtained from J.T. Baker, Xalostoc, Mexico. Compounds 1 (2-tert-Butyloxycarbonylamino-4-hydroxy-δ-valerolactam) and 2 (2-tert-Butyloxycarbonylamine-δ-valerolactam) were synthesized from our previous literature report [10].

The lipophilicity (capacity factor φ_0) determination was done by HPLC with a RP column (Agilent Zorbax C18, 150x46 mm, 5 μm particle size), at 1ml/min flow rate, detection 290 nm. The parameter φ_0 can be expressed as –log k´w/ S, with log k´w as the intercept of the curve log k´ vs % organic phase, and S as the slope of that curve [22].

Ethyl 2-(2-((*tert*-Butoxicarbonyl)amine)- δ-valerolactam-*N*-yl)acetate (ethyl 2-(3-((*tert*-Butoxycarbonyl)amine)-2-oxopiperidin-1-yl)acetate) (3). A solution of compound 2 (0.47 mmol) and NaH (1.87 mmol, 60% oil dispertion) in THF (4 ml) was cooled to 0°C and stirred at that temperature for 30 min. Ethyl bromoacetate (1.87 mmol) was added, brought to room temperature and allowed to react for 20 hr. The solution was quenched with $NaHCO_3$ saturated solution and extracted with AcOEt (3 x 10 ml). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo. Purification by flash chromatography (SiO_2, AcOEt/n-hexane, 1:1) resulted in product 3 as colorless oil. Yield: 83%; IR (υ_{max}, CH_2Cl_2, NaCl) cm^{-1}: 3398.57 (N-H secondary amide stretch), 2978.09 and 2873.94 (methyl C-H asym/sym stretch), 2939.52 (methylen C-H asym/sym stretch), 1747.51 (C=O carbonyl stretch in ester group), 1712.79 (C=O carbonyl stretch in carbamate group), 1658.78 (C=O carbonyl stretch in lactam ring), 1365.60 trimethyl C-H bend, 1246.02 and 1199.72 (O=C-O stretch in ester Bond), 1168.86 (C-O stretch in tertbutyl carbamate), 1072.42, 1026.13 and 995.27 (methylen lactam ring vibrations);^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (t, J=7.1 Hz, 3H), 1.44 (s, 9H), 1.65 (m, 1H), 1.97 (m, 2H), 2.49 (m, 1H), 3.38 (m, 2H), 3.91 (brd, J=17.2 Hz, 1H), 4.11 (m, 1H), 4.18 (m, 3H), 5.43 (brs, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.5, 21.2, 28.3, 28.7, 49.1, 49.4, 52.2, 61.6, 79.9, 156.3, 169.1, 170.8 ppm; MS (LMRS), m/z $C_{14}H_{24}N_2O_5Na$ (MNa+) calc: 323.3, found 323.1; $(M+Na\text{-}t\text{-}Butyl)^+$:266.9; $(M\text{-}t\text{-}Butyl)^+$:244.9; $(M+Na\text{-}Boc)^+$: 222.9; $(M\text{-}Boc)^+$: 200.9.

2-(2-*tert*-Butoxicarbonylamine-δ-valerolactam-*N*-yl) acetic acid (2-(3-*tert*-Butoxycarbonylamine-2-oxopiperidin-1-yl) acetic acid) (4). A solution of compound 3 (0.34 mmol) in EtOH-H_2O (1:1) (6 ml) was prepared. K_2CO_3 was added until pH 12. The mixture was stirred at room temperature for 24 h. The reaction was quenched with Brine solution and extracted once with AcOEt (5 ml). The aqueous phase was acidified with HCl 5% up to pH 3 and extracted with AcOEt (3 x 10 ml). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo, obtaining product 4, an amorphous white solid, used without further purification. Yield 86%; IR (υ_{max}, CH_2Cl_2, NaCl)

cm-^{1}: 3541.31(hydroxy group H bonded OH stretch), 2978.09 and 2873.94 (methyl C-H asym/sym stretch), 1747.51 (C=O carbonyl stretch in carboxylic acid group), 1712.79 (C=O carbonyl stretch in carbamate group), 1639.35 (C=O carbonyl stretch in lactam ring), 1369.46 (trimethyl C-H bend), 1257.59 (O=C-O stretch in carboxylic acid), 1157.29 (C-O stretch in tertbutyl carbamate), 1072.42, 1045.42, 1026.13 and 995.27 (methylen lactam ring vibrations); ^{1}H RMN (400 MHz, $CDCl_3$): δ 1.46 (s, 9H), 1.73 (m, 1H), 1.98 (m, 2H), 2.39 (m, 1H), 3.38 (m, 1H), 3.47 (m, 1H), 3.94 (d, J=17.4 Hz, 1H), 4.13 (m, 1H), 4.27 (d, J=17.4 Hz, 1H), 5.65 (brs, 1H), 8.30 (brs, 1H) PPM; ^{13}C RMN (100 MHz, $CDCl_3$): δ 21.4, 28.2, 28.7, 49.4, 49.6, 52.1, 80.2, 156.6, 171.6, 172.2 ppm; MS (LRMS) m/z, $C_{12}H_{20}N_2O_5$ (M^+) calc: 272.3, found 271.5; $(M+2H\text{-}Boc)^+$: 196.9.

General procedure for synthesis of 1*H*-benzimidazol-2-amine derivatives (5, 6 and 7). A suspension of methyl 1*H*-benzimidazol-2-ylcarbamate derivative (ABZ, FLU or FEB, 4.1 mmol for all of them) in HBr 24% (10 ml) was refluxed for 19 h. This was quenched with $NaHCO_3$ or NaOH, and then extracted with AcOEt (5 x 20 ml). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo.

6-(Propylthio)-1*H*-benzimidazol-2-amine (5). Purification by flash chromatography was done with AcOEt/ MeOH (10:1), obtaining product 5 as a light yellow amorphous solid. Yield 69% ; IR (υ $_{max}$, KBr) cm^{-1}: 3394.72 (aromatic primary amine N-H stretch), 3140.11 and 3078.39 (aromatic C-H stretch), 2962.66 and 2870.08 (methyl C-H asym/sym stretch), 2924.09 and 2758.21 (methylen C-H asym/sym stretch), 1666.50, 1643.35, 1566.20, 1531.48 and 1512.19 (aromatic ring stretch C=C-C), 1307.74 (aromatic tertiary amine, -imidazol- C-N stretch), 1292.31 (aromatic secondary amine –imidazol- C-N stretch), 1269.16 (aromatic primary amine C-N stretch), 921.97, 852.54 and 810.10 (aromatic C-H out of plane); ^{1}H RMN (400 MHz, DMSO-d_6): δ 0.95 (t, J=7.3 Hz, 3H), 1.50 (m, 2H), 2.78 (t, J=8.0 Hz, 2H), 6.27 (brs, 2H), 6.86 (d, J=8.0 Hz, 1H), 6.92 (d, J=8.0 Hz, 1H), 7.15 (s, 1H), 10.71 (brs, 0.8H) ppm; ^{13}C RMN (100 MHz, DMSO-d_6): δ 13.5, 18.2, 22.6, 37.8, 112.1, 115.3, 123.4, 124.4, 156.3, 156.4 ppm; MS (LRMS) m/z, $C_{10}H_{12}N_3S$ $(MH)^+$calc: 208.3, found 208.1; $(M\text{-}Propyl)^+$:164.9; $(M\text{-}Propyl\text{-}S)^+$: 133.0.

6-(4-Fluorobenzoyl)-1*H*-benzimidazol-2-amine (6). The Product was obtained as a white amorphous solid and used without further purification. Yield 65 % yield; IR (υ $_{max}$, KBr) cm^{-1}: 3437.15 (aromatic primary amine N-H stretch), 3186.40 (aromatic C-H stretch), 1670.35 (carbonyl conjugated with two aromatic rings), 1643.35 (aromatic ring stretch C=C-C, benzimidazole), 1600.92 (aromatic ring stretch C=C-C, phenyl 1,4 disubstituted), 1581.63 (aromatic ring stretch C=C-C, phenyl 1,4 disubstituted), 1554.63 (aromatic ring stretch C=C-C, benzimidazole), 1323.17 (aromatic tertiary amine, imidazol, C-N stretch), 1288.45 (aromatic secondary amine, imidazol, C-N stretch), 1238.30 (aromatic primary amine C-N stretch), 848.68 (aromatic C-H out of plane bend 1,4 disubstitution), 767.67 (aromatic C-H out of plane bend, benzimidazole ring); ^{1}H RMN (400 MHz, DMSO-d_6): δ 6.71 (s, 2H), 7.20 (d, J=8.24, 1H), 7.37 (t, J=8.9 Hz, 2H), 7.39 (dd, J=1.7, 1H), 7.54 (d, J=1.5, 1H), 7.77 (dd, J=5.6, 2H) ppm; ^{13}C RMN (100 MHz, DMSO-d_6): δ 112.3, 112.9, 115.6, 115.8, 123.8, 128.0, 132.4, 132.5, 135.9, 158.1, 163.2, 165.6, 194.5 ppm; MS (LRMS) m/z, $C_{14}H_{10}N_3FO$ $(M)^+$ calc: 255.08, found: 255.05; $C_{14}H_{11}N_3FO$ $(MH)^+$ calc: 256.08, found: 256.05; $C_8H_6N_3O^+$ calc:160.05, found: 160.10; $C_7H_4FO^+$ calc: 123.02, found: 123.10; $C_7H_7N_3^+$ calc:133.06, found: 133.10.

6-(Phenylthio)-1*H*-benzimidazol-2-amine (7). The residue was purified by flash chromatography first with AcOEt and then with AcOEt/ MeOH (10:1), obtaining product **7** as a white amorphous solid. Yield 87%; IR (υ $_{max}$, KBr) cm^{-1}: 3406.29 and 3344.57 (aromatic primary amine N-H stretch), 3074.53 and 3055.24 (aromatic C-H stretch), 1674.21 and 1581.63 (aromatic ring stretch C=C-C, phenyl), 1643.35 and 1554.03 (aromatic ring stretch C=C-C, benzimidazole), 1273.02 (aromatic primary amine C-N stretch), 867.97 and 813.96 (aromatic C-H out of plane, benzimidazole), 736.81 and 690.52 (aromatic C-H out of plane, phenyl); Raman spectroscopy (υ $_{max}$, KBr at 5%) cm^{-1}: 577 (C-S-C bend), 1134 (C=C aromatic ring stretching or C-S stretching), 1189 (C-N stretching), 1635 (C=C aromatic ring stretching); ^{1}H RMN (400 MHz, DMSO-d_6): δ 6.39 (s, 2H), 7.03 (dd, J=1.7, 1H), 7.06 (dd, J=1.2 Hz, 2H), 7.12 (t, J=7.4, 1H), 7.15 (d, J=8.0, 1H), 7.23 (s, 1H) 7.24 (d, J=8.3, 2H) ppm; ^{13}C RMN (100 MHz, DMSO-d_6): δ 125.7, 127.2, 129.5, 140.1, 156.9 ppm; MS (LRMS) m/z, $C_{13}H_{11}N_3S$ $(M)^+$ calc: 241.07, found: 241.05; $C_{13}H_{12}N_3S$ $(MH)^+$ calc: 242.07, found: 242.05; $C_7H_6N_3S^+$ calc:164.04, found: 164.05.

2-(2-*tert*-Butoxycarbonylamino-δ-valerolactam-*N*-yl)-*N*-(5-(propylthio)-3*H*-benzimidazol-2-yl) acetamide (2-(3-*tert*-Butoxycarbonylamino-2-oxopiperidin-1-yl)-*N*-(5-(propylthio)-3*H*-benzimidazol-2-yl) acetamide) (8). A solution of product 4 (0.37 mmoles), EDCI.HCl (0.73 mmoles) and HOBT (0.44 mmoles) in 1 ml of CH_2Cl_2 was stirred for 30 min at room temperature. Then, a solution of product 5 (0.44 mmoles) and TEA (0.518 mmoles) in 1 ml of CH_2Cl_2 was added, allowing to react for 19 h. The reaction was quenched with 5 ml of HCl 5% and extracted with AcOEt (3 x 10 ml). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo. Purification by flash chromatography was done with AcOEt/ MeOH (20:1), obtaining product 8 as a white amorphous solid. Yield 90% yield; IR (υ $_{max}$, KBr) cm^{-1}: 3292 (heterocyclic secondary amine N-H stretch), 2966 (methyl C-H asym/sym stretch), 2931 and 2871 (methylen C-H asym/sym stretch), 1702 (carbonyl in carbamate group C=O stretch), 1638 (carbonyl in lactam ring or amide between valerolactam and benzimidazole ring C=O stretch), 1571 (aromatic ring stretch C=C-C, benzimidazole), 1366 (tert butyl C-H asym/sym Bend), 1166 (C-O stretch in tertbutyl carbamate group), 1029 and 923 (methylene lactam ring vibrations); ^{1}H RMN (400 MHz, $CDCl_3$): δ 1.00 (t, J=8 Hz, 3H), 1.40 (s, 9H), 1.65 (m, 1H), 1.85 (m, 1H), 2.05 (m, 1H), 2.42 (m, 1H), 2.87 (t, J=8 Hz, 2H), 3.43 (m, 1H), 3.58 (m, 1H), 4.17 (m, 2H), 4.51 (m, 1H), 6.03 (brd, 1H), 7.24 (d, J=8 Hz, 1H), 7.36 (d, J=8 Hz), 7.54 (s, 1H), 9.13 (brs, 1H) ppm; ^{13}C RMN (100 MHz, $CDCl_3$): δ 13.4, 21.1, 22.6, 28.0, 28.3, 37.4, 49.9, 51.7, 51.8, 79.7, 114.3, 115.9, 125.8, 130.2, 147.1, 156.2, 169.2, 171.5 ppm; MS (HRMS) m/z, $C_{22}H_{31}N_5O_4SNa$ $(MNa)^+$ calc: 484.20, found: 484.3; $C_{22}H_{31}N_5O_4S$ $(MH)^+$ calc: 462.22, found 462.40.

2-(2-*tert*-Butoxycarbonylamino-δ-valerolactam-*N*-yl)-*N*-(5-(4-fluorobenzoyl)-3*H*-benzimidazol-2-yl) acetamide (2-(3-*tert*-Butoxycarbonylamino-2-oxopiperidin-1-yl)-*N*-(5-(4-fluorobenzoyl)-3*H*-benzimidazol-2-yl) acetamide) (9). A solution of product 4 (0.33 mmoles), EDCI.HCl (0.50 mmoles) and HBTU (0.50 mmoles) in 2 mL of DMF was stirred for 30 min at room temperature. Then a solution of product 6 (0.40 mmoles) and TEA (0.462 mmoles) in 2 ml of CH_2Cl_2 was added, allowing to react for 20 h. The reaction was quenched with 5 ml of a saturated solution of $NaHCO_3$ and HCl 5% was added until pH 5. The extraction was done with ethylic eter (3 x 10 mL) and finally with $CHCl_3$ (2 x 10 ml). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo. Purification by flash chromatography was done with CH_2Cl_2 first and then with CH_2Cl_2 5 % MeOH, obtaining product 9 as a white amorphous solid. Yield 49%; IR (υ_{max}, KBr) cm^{-1}: 3356.14 (heterocyclic secondary amine N-H stretch), 3043.67 (aromatic C-H stretch), 2974.23 (methyl C-H asym/sym stretch), 2931.80 (methylene C-H asym/sym stretch), 1716.65 (carbonyl in carbamate group C=O stretch), 1639.49 (carbonyl group in lactam ring, amide link between valerolactam and benzimidazole rings or carbonyl conjugated with aromatic rings), 1612.49 (aromatic ring stretch C=C-C, benzimidazole), 1597.06 and 1566.20 (aromatic ring stretch C=C-C, 1,4 disubstituted ring), 1365.60 (tertbutyl C-H asym/sym bend), 1303.88 (aromatic tertiary amine C-N stretch, imidazol), 1288.45 (aromatic secondary amine C-N stretch, imidazol), 1165.00 (C-O stretch in tertbutyl carbamate group), 1072.42, 1045.72 and 1026.13 (methylen lactam ring vibrations), 852.52 (aromatic C-H out of plane bend 1,4 disubstituted -para- ring), 775.38 (aromatic C-H out of plane bend, benzimidazole ring); ^{1}H NMR (400 MHz, DMSO-d6): δ 1.38 (m, 9H), 1.90 (m, 5H), 3.17 (d, J= 5.3 Hz, 1H), 3.42 (m, 2H), 4.00 (m, 1H), 4.23 (dd, J=16.9 Hz, 2H), 6.98 (m, 1H), 7.40 (t, J= 8.7 Hz, 2H), 7.57 (s, 1H), 7.81 (m, 2H) ppm; ^{13}C NMR (100 MHz, DMSO-d6): 21.5, 28.2, 28.7, 29.5, 49.1, 49.3, 50.5, 51.1, 78.3, 115.8, 116.0, 132.7, 155.9, 170.3 ppm; MS (HRMS) m/z, $C_{26}H_{28}FN_5O_5Na$ $(MNa)^+$ calc: 532.1972, found: 532.1967; $C_{26}H_{29}FN_5O_5$ $(MH)^+$ calc: 510.2153, found: 510.2147.

2-(2-*tert*-Butoxycarbonylamino-δ-valerolactam-*N*-yl)-*N*-(5-(Phenylthio)-3*H*-benzimidazol-2-yl) acetamide (2-(3-*tert*-Butoxycarbonylamino-2-oxopiperidin-1-yl)-*N*-(5-(Phenylthio)-3*H*-benzimidazol-2-yl) acetamide (10). A solution of product 4 (0.51 mmoles), DEPBT (1.03 mmoles) in 2 mL of THF was stirred for 30 min at room temperature. Then a solution of product 7 (0.77 mmoles) and DIPEA (1.03 mmoles) in 2 ml of THF was added, allowing to react for 19 hr. The reaction was quenched with 10 ml of a saturated solution of $NaHCO_3$. A first extraction with ethyl acetate was done (3x10 ml). The organic phase was extracted with a saturated solution of NaCl. The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuum. Purification by flash chromatography was done with a mixture of $CHCl_3$: Hexane (2:1) with 6% of methanol, obtaining product 10 as a white amorphous solid. Yield: 88%; IR (υ_{max}, KBr) cm^{-1}: 3221.12 (heterocyclic secondary amine N-H stretch), 3055.24 (aromatic C-H stretch), 2974.23 (methyl C-H asym/sym stretch), 2931.80 and 2866.22 (methylene C-H asym/sym stretch), 1708.93 (carbonyl in carbamate group C=O stretch), 1678.07 (carbonyl group in amide between valerolactam and benzimidazole ring), 1635.64 (carbonyl group in lactam ring), 1581.63 and 1500.62 (aromatic ring stretch C=C-C), 1365.60 (tertbutyl C-H asym/sym bend), 1280.73 (aromatic secondary amine C-N stretch, imidazol), 1165.00 (C-O stretch in tertbutyl carbamate group), 1072.42, 1049.28 and 1026.13 (methylen lactam ring vibrations), 867.97 and 810.10 (aromatic C-H out of plane bend), 740.67 and 690.52 (aromatic C-H out of plane bend, phenyl ring). Raman spectroscopy (υ_{max}, KBr at 5%) cm^{-1}: 576 and 601 (C-S-C bend), 1466 (C-N stretching), 1637 (C=C aromatic ring stretching); ^{1}H NMR (400 MHz, DMSO-d6): δ 1.38 (m, 9H), 1.84 (m, 4H), 3.41 (m, 2H), 4.01 (m, 1H), 4.21 (dd, J=16.9 Hz, 2H), 6.99 (d, J=8.7 Hz, 1H), 7.13 (d, J= 7.2 Hz, 2H), 7.17 (t, J=7.4 Hz, 1H), 7.21 (dd, J=1.7 Hz, 1H), 7.29 (t, J=7.3 Hz, 2H), 7.49 (d, J=8.3 Hz, 1H), 7.57 (s, 1H) ppm; ^{13}C NMR (100 MHz, DMSO-d6): 21.5, 28.2, 28.7, 43.3, 51.1, 78.3, 79.6, 126.3, 128.1, 129.7, 138.9, 170.2 ppm; MS (HRMS) m/z, $C_{25}H_{29}N_5O_4SNa$ $(MNa)^+$ calc: 518.1838, found: 518.1832; $C_{25}H_{30}N_5O_4S$ $(MH)^+$ calc: 496.2019, found: 496.2013.

2.2. Biological Assays

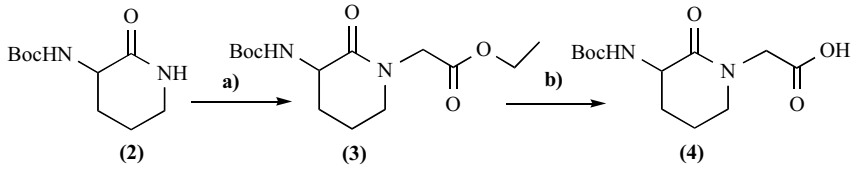
2.2.1. In Vivo Drug Screening

The *in vitro* and *in vivo* anthelmintic activity of compounds was determined using the *N.brasiliensis* L4 physiology-based bioassays, standardized with albendazole, febendazole, and levamizole (reference drugs) described previously [10], [11]. Animal protocol was in conformity with Uruguayan Law No. 18.611(http:/www.presidencia.gub.uy/web/leyes/2009/EC1395.pdf) and harmonized with The Canadian Guidelines on Animal Care. The experimental protocol of the study was reviewed and approved by IACUC of Facultad de Química - Universidad de la República, Uruguay, under approval number 06-05-09 (http:/www.chea.udelar.edu.uy).

The larvae of *N. brasiliensis* undergo a migratory phase [23] through the rat's lungs before entering the small intestine as L4 parasites (72 h post infection) [24]. Since the adult parasites are naturally expelled from the host's gut [25], we have demonstrated previously [11] the use of L4 stage of *N. brasiliensis* (instead of adult parasites) for preliminary *in vivo* drug screening using albendazole, febendazole, and levamizole as reference drugs. Briefly, groups of rats were subcutaneously infected with 5000 L3 stage larvae of *N. brasiliensis*. The animals were dosed orally with drugs in gelatin microcapsules at 5 h post-infection (p.i.). Three groups of animals were dosed orally (5, 19 and 64 mg/kg body weight) with a valerolactam derivative (product 1), and one group served as untreated control. The animals were euthanized and the intestines were dissected out 72 h p.i. and were examined with the Baermann method for L4 determination. The numbers (mean ± standard error of the mean) of L4s recovered were determined for each dosed group, and the results were analyzed using ANOVA and Tukey's post-run test ($p < 0.05$).

2.2.2. In Vitro Drug Screening

Briefly, Wistar rats were infected subcutaneously with 5000 L3 larvae of *N. brasiliensis* and euthanized by cervical



Scheme (1). Reaction conditions: **a**) NaH, ethyl bromoacetate, THF, 20 hs (3, 83%); **b**) K2CO3, EtOH:H2O (1:1), 22 hs (4, 88%).

dislocation 72 h (p.i.). L4 parasites were recovered from intestines, washed, and kept in a 24-well tissue culture plate containing 1.8 ml culture medium and 50 L4s suspended in 0.2 ml medium per well. Samples in each well were dissolved and diluted to the desired final concentration by adding 10 µl dimethylsulphoxide (DMSO) (0.5% v/v per well). Plates were incubated at 37º C and the number of dead parasites read under an inverted microscope on day five of culture. Readings were corrected against DMSO controls. Results were analyzed by ANOVA and Tukey´s post-run test ($p < 0.05$). The mean effective concentration (EC_{50}) — corresponding to 50% dead parasites— was calculated for each treatment by the probit method at a confidence level of 95% by means of Prism GraphPad 5.00 software (2008, San Diego, USA), and later confirmed experimentally.

2.2.3. Ex Vivo Drug Diffusion Experiments

Ex vivo diffusion experiments of product 1, ABZ and hybrid compound 8 into *N. brasiliensis* were performed according to literature [26] with minor modifications. Freshly collected L4 parasites (0.4 g wet weight) were incubated at 37º C in 4 ml phosphate buffer saline (PBS) containing each drug at the same final concentration (40 µM) for time periods of 1.5 or 6 h.

Preparation and Clean Up of Samples for Analysis

Parasites were washed rapidly 3 times with PBS, homogenized (Cole Parmer CPX 600 ultrasonic homogenizer, Vernon Hills, Illinois, USA) with 5 volumes of PBS (3 times for 30 sec at 0º C), centrifuged at 3500 rpm for 20 min and the supernatant evaporated to dryness. The residue was dissolved in 1 ml of water:methanol (80:20) and injected into C_{18} cartridges (Sep-Pack ®, Waters, Milford, Massachusetts, USA) previously conditioned. The cartridge was flushed with 10 ml of water and eluted with 3 ml of methanol, evaporated and the residue dissolved with 100 µl of acetonitrile prior to HPLC analysis. For compound 1, the PBS suspension was extracted with 5 volumes of ethyl acetate, centrifuged at 3500 rpm for 20 min and the organic phase evaporated to dryness, dissolved in 100 µl of methanol prior to HPLC analysis.

Protein Concentration

Parasite protein concentration was determined according to Smith [27] with a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA).

HPLC Analysis and Method Validation

Quantification of drugs was done by spiking known amounts in parasite material and repeating the whole process described before (by triplicate). Recovery of compounds was estimated by comparing the peak areas from spiked parasite material samples with the areas resulting from direct injections of standards in methanol. Untreated parasite material (control) was incubated under the same conditions. HPLC analysis was set up using a C_{18} column (Phenomenex, particle size 5µm, 150 x 4.6 mm) at a flow rate of 1.00 ml/min. A mobile phase of water:acetonitrile (80:20) was used for compound 1 (retention time 3.5 min) with detection at 210 nm. Analysis of ABZ and compound 8 were carried out following the method described previously, with minor modifications [28]. The mobile phase was acetic acid (1%): acetonitrile: methanol (40:30:30), with retention times of 4.7 and 10.7 min (ABZ and compound 8, respectively), detection at 290 nm.

Analytical procedure was validated according to the following criteria: linearity was established from calibration curve using least-squares linear regression analysis and correlation coefficient (r) was calculated; accuracy and precision was evaluated by processing replicates of drug-free samples spiked with known amounts of product 1 (n=6) and expressed as relative standard deviation (RSD); the limit of drug quantification (LOQ) was established with corresponding blanks, measuring the baseline noise at the corresponding retention time of the analyte, and this value plus six standard deviations was defined as the theoretical quantification limit.

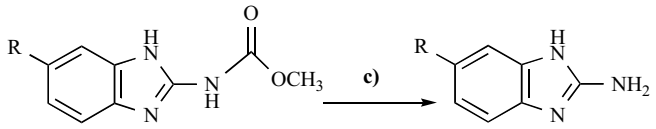
The total amount of compound inside the parasites was expressed as nmol/ 100 mg of protein.

3. RESULTS AND DISCUSSION

The anthelmintic valerolactam moiety was synthesized as described in Scheme **1**, starting from L ornithine cyclodehydration with alumina by refluxing in toluene [29]. Cyclized ornithine was protected in the amine group at position 2 with di-tert-butyl-dicarbonate (Boc2O). A nucleophilic substitution in position 6 with ethyl bromoacetate, followed by basic hydrolysis with K2CO3 resulted in compound 4 (valerolactam acetic acid moiety).

The amine-benzimidazole subunits were obtained from commercial benzimidazole carbamates (Scheme **2**) albendazole, flubendazole and febendazole (ABZ, FLU, FEB, respectively), by means of acid hydrolysis with hydrobromic acid of the methylcarbamate group [30].

The hybrid compounds (8-10) were obtained by amide coupling reactions between acid 4, and 2-amine-benzimidazoles (5-7), using different coupling agents: 1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide hydrochloride (EDCI.HCl), Hydroxybenzotriazole (HOBT), O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro - phosphate (HBTU) and 3-(Diethoxy-phosphoryloxy)-3H-benzo[d][1,2,3] triazin-4-one (DEPBT), in dichloromethane, tetrahydrofuran (THF) or dimethylformamide (DMF). Compounds (8-10) were obtained in good yields (49-90 %).



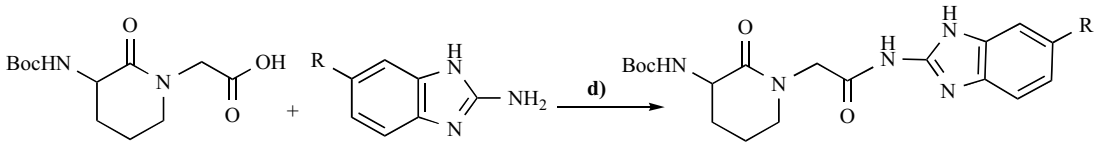
ABZ, FLU, FEB

ABZ, R= - $S(CH_2)_2CH_3$
FLU, R= -CO-4-FPh
FEB, R= -SPh

(5-7)

5, R= - $S(CH_2)_2CH_3$
6, R= -CO^{-4}-FPh
7, R= -SPh

Scheme (2). Reaction conditions: **c**) Reflux in HBr 24%, 19 hs for 5-7 benzimidazole amine derivatives (5, 69%; 6, 65%; 7, 87%).



(4) **(5-7)** **(8-10)**

5, R= -$S(CH_2)_2CH_3$
6, R= -CO^{-4}-FPh
7, R= -SPh

8, R= -$S(CH_2)_2CH_3$
9, R= -CO^{-4}-FPh
10, R= -SPh

Scheme (3). Reaction conditions: d) 8: EDCI, HOBT and TEA in CH2Cl2, 19 hs (7, 90%). 9: EDCI, HBTU and TEA in DMF, 20 hs (8, 49%). 10: DEPBT and DIPEA in THF, 19 hs (9, 88%).

Table 1. ***In Vivo*** **Anthelmintic Activity Using** ***N. Brasiliensis*** **L4 in Rat Bioassay. Number of L4 Parasites Recovered 72 h p.i. from the Intestines of Untreated and Treated Rats with Valerolactam Derivative (Product 1) at 5, 19 and 64 mg/kg.**

Product 1				
Group	**Untreated (Control)**	**Treated with 5 mg/kg**	**Treated with 19 mg/kg**	**Treated with 64 mg/kg**
	3510	3160	2982	2360
	4017	2920	1160	2580
	4220	2800	2660	1320
	3880	3440		1940
	4422			
Mean	4010*	3080	2267*	2050*
SD	347	283	972	554
Efficacy (% ± SEM)	---	Inactive	43.5 ± 16.8	48.9 ± 9.1

*Means show significant differences (P<0.05) with not treated group. Treated groups do not show significant differences (P<0.05) between them.

Preliminary in vivo studies of anthelmintic activity in N. brasiliensis rat model showed very low efficacy in the valerolactam treated groups of rats at the assayed doses. The efficacy (%) between those treated groups did not show a dose dependent effect (43.5 and 48.9 % for 19 and 64 mg/kg of dose, respectively) (Table **1**).

The lipophilicity (capacity factor φ_0) of the hybrid compounds synthesized, valerolactam intermediates, and benzimidazole carbamate analogs determined by the HPLC method (reverse phase system) are shown in Table **2**. The valerolactam-benzimidazole hybrids presented capacity factors (φ0) similar to those obtained for commercial benzimidazole analogs, and 1.5 times higher compared with valerolactam moiety (compound 2).

The *in vitro* anthelmintic activity (Table **2**) of hybrid compounds (8-10) proved to be greater (EC_{50} of $6.08x10^{-4}$, $5.76x10^{-1}$ and $3.87x10^{-6}$ nM for 8, 9, and 10 respectively) than their corresponding benzimidazole carbamate analogs (EC_{50}: 340, 100 and 120 nM for ABZ, FLU and FEB, respectively).

It is noted that *in vitro* activities of nanomolar range were also reported for some known anthelmintics using different target helminths, as ivermectin [31], or the more recent anthelmintic emodepside [32].

On the other hand, the analytical method for diffusion studies, particularly of compound 1, was validated according to the following parameters: recovery, 75 %; accuracy (RSD), 2.9%; LOQ, 0.1µg/ml; linearity (r): 0.9990.

Table 2. Anthelmintic Activity (EC_{50}) in *N. Brasiliensis* L4 *In Vitro* Bioassay, and Lipophilicity Factor (φ_0) of: Valerolactam Derivatives (1-4), Hybrid Compounds (8-10) and Commercial Anthelmintic Benzimidazoles.

Product	EC_{50} ± SEM (nM) [a]	Lipophilicity Factor φ_0 [b]
1	2.00×10^{-1} [c]	21.6
2	2.20×10^{-1} [c]	30.8
3	$(5.00 \pm 1.66) \times 10^{-3}$	51.2
4	$(2.50 \pm 0.80) \times 10^{2}$	---
ABZ	$(3.40 \pm 0.80) \times 10^{2}$	58.0
8	$(6.08 \pm 1.55) \times 10^{-4}$	58.8
FLU	$(1.00 \pm 0.10) \times 10^{2}$	50.6
9	$(5.76 \pm 2.18) \times 10^{-1}$	56.2
FEB	$(1.20 \pm 0.20) \times 10^{2}$	62.5
10	$(3.87 \pm 0.37) \times 10^{-6}$	67.1

[a]The mean effective concentration (EC_{50}) —corresponding to 50% dead parasites *N. brasiliensis* L4— was calculated for each treatment (≥ 5 replicates) by the probit method at a confidence level of 95% [10]. SEM: Standard Error of the Mean.
[b]Determined experimentally by HPLC RP [22].
[c]Previously reported by us [10].
ABZ: albendazole, FLU: flubendazole, FEB: febendazole.

Comparative *ex vivo* diffusion studies show that valerolactam derivative 1 did not penetrate parasite barrier, even at 6 h of incubation time. However, ABZ and its hybrid compound 8 showed intraparasity concentrations of 249 and 196 nmol/ 100 mg of protein, respectively at 6 h, under the same experimental conditions (not shown).

4. CONCLUSION

Even when valerolactam derivatives showed extrordinary *in vitro* anthelmintic activity (nanomolar range), the lack of *in vivo* activity using *N.brasiliensis* physiology-based assay was explored. By means of comparative studies of *ex vivo* intraparasitary diffusion, it was demonstrated that valerolactam derivatives did not penetrate parasite barriers (under experimental conditions assayed).

Additionally, the hybridization strategy was useful in two ways. Firstly, those novel hybrid compounds also showed extraordinary *in vitro* anthelmintic activities. Secondly, the hybridization strategy allowed an increase in bioavailability into parasites, by means of the improvement of physicochemicals properties, such as lipophilicity.

In this communication we have demonstrated the usefulness of integrating the study of bioavailability into target parasite jointly with physiology-based assay, as a more appropriate way for preliminary screening of anthelmintic, before proceeding to in *vivo* bioassays (efficacy and pharmacokinetic studies).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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