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Original article

Synthesis and antitumour evaluation of peptidyl-like derivatives containing the 1,3-benzodioxole system

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Abstract

In the scope of a research program aiming at the synthesis and pharmacological evaluation of novel possible antitumour prototype compounds, we described in this paper the synthesis of peptidyl-like derivatives containing the 1,3-benzodioxole system. The proliferation inhibitors tested against tumour cell lines identified the derivatives tyrosine (**4f**) and lysine (**4g**) as the most active among them, presenting IC_{50} values in micromolar range and are more active than Safrole. For the study on the embryonic development, Safrole did not show any selectivity in this latter assay, which indicates that Safrole acts as a 'cell cycle-nonspecific' inhibitory agent. However, compound **4f** presented a fair antimitotic effect, mainly on third cleavage and blastulae stages (38% and 1.7% of normal development, at 10 μg/mL), suggesting a time-dependent activity and a 'cell cycle-specific' agent action. Neither derivatives revealed hemolytic action in assay with mouse erythrocytes.

Keywords: 1,3-Benzodioxole; Peptidyl-like derivatives; Cytotoxicity; Sea urchin embryos; Erythrocyte hemolysis

1. Introduction

According to the WHO, cancer is an important health problem that claims the lives of more than seven million people worldwide on an annual basis [1]. The P-glycoprotein (P-gp) has been held responsible for the effluxes of hydrophobic anticancer agents including paclitaxel and docetaxel, discussing about the occurrence of Multiple Drug Resistance (MDR) [2,3]. Development and optimisation of new cancer treating drugs is a must.

Safrole (1,3-benzodioxole-5-yl), from sassafras oil (*Ocotea pretiosa* Mer., Lauraceae), is an abundant Brazilian natural product that demonstrates interesting functionality and chemical reactivity suggesting its use as an efficient and versatile natural *synthon* [4–6]. The methylenedioxy core can be identified in several bioactive natural anticancer agents, as etoposide, tenoposide and podophyllotoxin, among others [7,8]. Jurd et al. [9] have reported a series of simple 6-benzyl-1,3-benzodioxoles, structurally related to podophyllotoxin, as more potent antitumour in vivo than the prototype. In addition, the SAR informations for podophyllotoxin and analogues have showed that converting the methylenedioxy unit to the corresponding methoxy/hydroxy group dramatically reduced the antitumour activity [10]. Two recent publications cite references describing the importance of 1,3-benzodioxole ring with antitumour properties. Xia et al. described that introduction of the methylenedioxy moiety for 2-amino-chalcone led to

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enhanced cytotoxic activity against multidrug-resistant KB carcinoma cells, compared with the unsubstituted analogue [11]. Hence, Micale et al. [12] reported that 1,3-benzodioxole derivatives exhibited in vitro tumour growth inhibition activity, which led to 6-(4-aminobenzoyl)-1,3-benzodioxole-5-acetic acid methyl ester as the most active compound of this series. More recently, novel analogues of podophyllotoxin and thuriferic acid containing different heterocyclic moieties, such as furan, thiophene and carbazole ring systems, as well as the less polar naphthalene moiety substituting the benzodioxole system of the prototype have been evaluated which revealed that benzodioxole system is an important requirement for antitumour activity for these natural lignan, being the bioisoster ring derivatives less active than the prototype [13].

According to carcinogenesis and metabolism studies, Safrole is metabolically activated with the electrophilic intermediates that bind with cellular DNA, to form adduct as guanine derivatives [14]. In addition, recently it was reported that Safrole induced an increase in Ca^{2+} levels in PC3 prostate cancer cell, with decrease in cell viability in a concentration-dependent manner [15].

Antitumour drugs that present reduced toxicity have been developed by linking them to small peptides or amino acids residues [16]. It is supposed that, the conjugated peptide, in these drugs serves as a substrate for designated enzymes that are produced and secreted preferentially by tumour tissue [17]. The rules for the peptidyl-prodrugs on antitumour activity have not yet fully established. Actually, some approaches have shown the use of specific sequence of complementary amino acids for tumour-associated enzymes, with the objective for rational selection of the peptidyl residues to be conjugated as tumour recognition moiety, such as a plasmin system (an inactive pro-enzyme plasminogen form) [18,19]. However, this strategy does not demonstrate to be absolute and new prediction for selection of these tumour recognition moieties is necessary.

In view of the available information on Structure–Activity Relationships (SAR) considering the potential of ring system from Safrole, in previous works we undertook the synthesis of compounds exploring five and six positions in aromatic ring from 1,3-benzodioxole core. In vivo assays showed that, some 1,3-benzodioxole peptidyl derivatives were able to inhibit sarcoma S-180 tumour at 35 mg/kg with low toxicity when compared to intermediary 6-allyl-benzo[1,3]dioxole-5-yl-amine that was toxic and lethal at the same dose [20]. Our results demonstrate that the linking of 1,3-benzodioxole core to an amino acid moiety reduced the intrinsic toxicity.

During our continuous effort aiming the synthesis and pharmacological evaluation of novel possible antitumour compounds, we began to optimize the 1,3-benzodioxole peptidyl derivatives by chemical modification at 5-position on aromatic ring to obtain new SAR data. It is well documented that the toxicophore unit from Safrole devils in C-5 moiety, with formation of diepoxide species (butadiene dimers) [21]. Then, we have decided introducing some lipophilic or basic protected amino acids at 5-position. Besides, in order to investigate the importance of nitro group for the cytotoxic activity, some compounds were substituted in 6-position by a nitro group. It is well known that nitro group could act to form oxygen species reactive or as internal catalyst and to promote chemical breakdown in bioactive compounds [22]. To exploit the activities of the benzodioxole system, we also describe here, the in vitro evaluation of proliferation inhibitors against tumour cell lines, embryonic development with sea urchin (*Lytechinus variegatus*) embryos and hemolytic action for peptidyl-like derivatives containing the 1,3-benzodioxole system.

2. Results and discussion

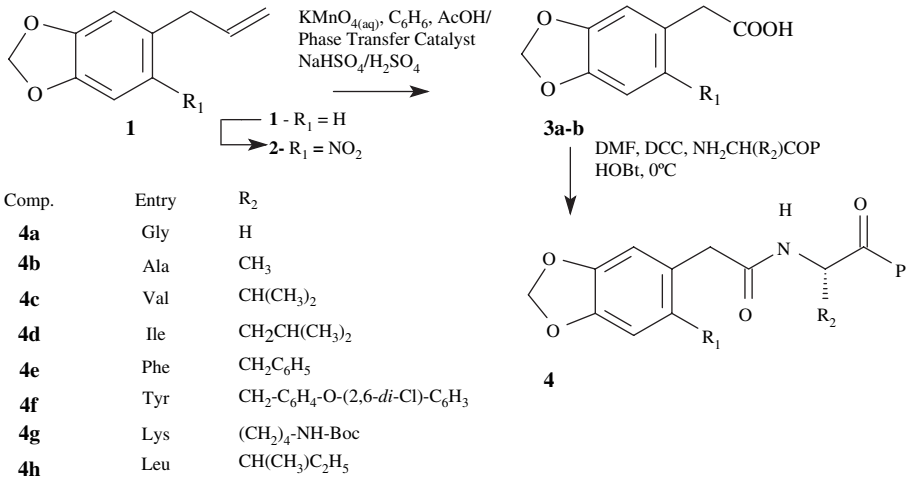
2.1. Chemistry and spectroscopy mean

Among several obvious synthetic route to obtain the peptidyl-like derivatives **4a–h**, we decided to explore the 5-position on aromatic ring of Safrole. Besides, the compounds **4d–g** were substituted in 6-position, in order to investigate the importance of nitro group for the cytotoxic activity. Safrole (**1**) and 5-allyl-6-nitro-benzo[1,3]dioxole (**2**) were used to obtain the intermediate product (6-nitro-benzo[1,3]dioxole-5-yl) acetic acid (**3a**) or (6-*H*-benzo[1,3]dioxole-5-yl) acetic acid (**3b**). The condensation reactions with L-α-amino acids (*S* enantiomer) were accomplished by employing the classical method of peptide synthesis (Scheme 1) [23]. DCC (dicyclohexylcarbodimide) and HOBt (hydroxybenzotriazole) were used to prepare the series of peptidyl derivatives of Safrole [20]. Side chains of lysine were protected by a *tert*-butoxycarbonyl and tyrosine by 2,6-di-chlorobenzyl ether.

Structures of these compounds were characterized using IR, ^{1}H NMR and elemental analyses. Physical and spectral data are given in Tables 1 and 2. The common characteristic signals of all compounds showed a characteristic N–H stretching vibration around 3193 and 3387 cm^{-1}, overlaps C = O stretching, amide first band around 1627 and 1671 cm^{-1} and C–O–C symmetrical stretching appears near 1020–1088 cm^{-1}. In the ^{1}H NMR spectra, methylene protons displayed as one singlet at 4.21–3.35 ppm and at 6.02–6.27 ppm attributed to the methylenedioxy group. The methin protons of the chiral centre showed resonances at 3.35–4.68 ppm, and for compounds **4c** and **4d**, the other methin protons gave a multiplet at 1.59–2.22 ppm in each case. The NH_2 signals appear as two singlets or only one singlet at 6.95–7.42 ppm and NH protons resonated as a singlet or a doublet non-equivalents at 7.98–8.60. The *tert*-butoxycarbonyl peak appeared as a large and broad singlet at 1.10 ppm and the 2,6-di-chlorobenzyl ether displayed a multiplet at 7.45–7.56 ppm.

2.2. Cancer cell assays

The peptidyl-like derivatives were evaluated against four tumour cell lines (MDA/MB-435, HCT-8, HL-60, and SF-295), using a previously described MTT assay. Table 3 summarizes the IC_{50} data (μM) for antitumour activity. The results indicated that compounds **4a** (glycine), **4f** (tyrosine) and **4g** (lysine) have comparable and significant activities and are



Comp.	Entry	R_2
4a	Gly	H
4b	Ala	CH_3
4c	Val	$CH(CH_3)_2$
4d	Ile	$CH_2CH(CH_3)_2$
4e	Phe	$CH_2C_6H_5$
4f	Tyr	CH_2-C_6H_4-O-(2,6-*di*-Cl)-C_6H_3
4g	Lys	$(CH_2)_4$-NH-Boc
4h	Leu	$CH(CH_3)C_2H_5$

P = OEt: **4a**; P = NH_2: **4b-h.** R_1 = H: **3a**, **4a-c**,**h**. R_1 = NO_2:**3b**, **4d-g**.

Scheme 1.

the most potent proliferation inhibitors in this series, with best IC_{50} values of 4.3 (HCT-8), 8.5 (HCT-8) and 5.4 μM (HL-60), respectively. The growth of cancer cell lines was also inhibited by Safrole, with IC_{50} values of 32.6 μM for SF-295 and 36.1 μM for HCT-8 cells. The peptidyl-like derivatives containing the nitro group were the most potent than non-nitrated analogues. The other compounds studied in this series were not able to inhibit cell growth in this assay ($IC_{50} > 50$ μM). The differences in potency among the derivatives and Safrole shown here may indicate that their antiproliferative effects are not solely due to the reactive benzodioxole system, since their activity was influenced by the pattern of nitro group in aromatic ring. Regarding the use of different amino acids, the bulk and basic groups in the side chain (like lysine and tyrosine) can be a recognition pattern to answer the increase of the cytotoxic activity.

Despite Safrole being classified as a weak hepatocarcinogen in rodents and possibly in human [24], it has epoxy structures which are important in many compounds with physiological activity [25]. The carcinogenicity of Safrole is usually thought to be caused by Safrole–DNA adducts formation [26]. After Safrole exposure, Safrole–DNA adducts have been found in many tissues in animal models and human [27]. In addition, potent effects of Safrole oxide were observed on vascular endothelial cells (VECs), suggesting that Safrole oxide might be promising for angiogenesis inhibition [28]. Recently, studies suggest that Fas/FasL pathway might be activated by Safrole oxide and then mediated the apoptosis of tumour cell lines, and this may constitute a potential mechanism in the mediation of anticancer drug-induced apoptosis [29].

2.3. Antimitotic activity

The antimitotic activity was performed on the embryonic development of fertilized sea urchin eggs assay and is shown in Table 4. Safrole inhibited the cleavages of sea urchin eggs in a concentration-dependent manner. This inhibition is significant since the first cleavage for both tested concentrations (10 and 100 μg/mL). In contrast, the peptidyl-like derivatives (**4a**, **4c** and **4g**) presented a fair antimitotic effect, mainly on third cleavage and blastulae stages and at the highest tested concentrations. Compound **4f** presented inhibitory activity at both tested concentrations but it was not able to inhibit the first cleavage, while compound **4e** completely inhibited the development of sea urchin eggs at the highest tested concentration (100 μg/mL) since the first cleavage. The remaining tested compounds (**4b**, **4d** and **4h**) were not able to inhibit mitosis on the embryos in this assay. Jacobs et al. [30] have considered very active a substance that promotes 100% of inhibition in this assay at a concentration of 16 μg/mL or less. Based on this consideration, **4f** was the most active compound tested, whereas it was able to inhibit almost any division at the blastulae phase.

The sea urchin egg development possesses some peculiarities, making possible to suggest how the tested substances acted. The inhibition at the first cleavage is related to DNA and/or protein synthesis or microtubule assembly, once RNA synthesis is very slow or absent after fertilization. At this time, the rapid increase in the rate of protein synthesis is largely due to the recruitment of maternal mRNA into polysomes [31]. However, when a compound blocks microtubule assembly, clear spots corresponding to nucleus duplication can be observed in the cytoplasm. Since cells treated with Safrole (**1**) and compound **4e** presented a homogeneous cytoplasm, this process appears not to have been affected [32]. Hence, these compounds might affect DNA and/or protein synthesis. On the other hand, based on the present data it is not possible to affirm whether the delayed activity of compounds **4a**, **4c**, **4f** and **4g** is only a result of the chemical properties of these compounds or a different target though the antimitotic activity shown by these peptidyl derivatives was in agreement with Batra et al. [33] who showed that compounds containing methylenedioxy unit are capable to inhibit the assembly of microtubules. Moreover, the benzodioxole system also prevented microtubule formation in the ovaries of *Drosophila melanogaster* [34].

Table 1
^{1}H NMR spectral data of newly synthesized derivatives

Compound	^{1}H NMR (DMSO-d_6) δ
4a	1.19 (t, $J = 7.19$ Hz, 3H, $CH_3\beta$), 3.82 (d, $J = 5{,}7$ Hz, 2H, $CH_2\alpha$), 3.85 (s, 2H, $CH_2\alpha'$), 4.08 (q, $J = 7.19$ Hz, 2H, $CH_2\alpha''$), 6.21 (s, 2H, $CH_2$2), 7.06 (d, $J = 1.50$ Hz, 1H, Ar-4), 7.49 (d, $J = 8.00$ Hz, 1H, Ar-6), 7.61 (s, 1H, Ar-7), 8.33 (broad, 1H, NH-10).
4b	1.19 (t, $J = 11.09$ Hz, 3H, $CH_3\alpha$), 3.35–3.55 (m, 1H, CHα), 4.16 (s, 2H, $CH_2\gamma'$), 6.06 (s, 2H, $CH_2$2), 6.88 (d, $J = 8.39$ Hz, 1H, Ar-4), 7.15 (s, 1H, NH-13), 7.49 (d, $J = 8.39$ Hz, 1H, Ar-6), 7.75 (s, 1H, Ar-7), 8.35 (s, 1H, NH-11).
4c	0.91 (d, $J = 7.19$ Hz, 3H, $CH_3\Delta$), 2.05–2.22 (m, 1H, CHγ), 3.82 (d, $J = 6.50$ Hz, 1H, CHβ), 3.83 (s, 2H, $CH_2\alpha$), 5.32 (s, 1H, NH_2-12), 6.07 (s, 2H, $CH_2$2), 6.88 (d, $J = 8.09$ Hz, Ar-4), 7.49 (d, $J = 7.49$ Hz, Ar-6), 7.75 (s, 1H, Ar-7), 8.47 (s, 1H, NH-10).
4d	0.79 (dd, $J = 7.49$ Hz, $J = 6.8$ Hz, 3H, $CH_3\Delta$), 0.83 (d, $J = 5.89$ Hz, 3H, $CH_2\gamma$), 1.02–1.09 (m, 1H, $CH_2\gamma$), 1.18–1.3 (m, 1H, $CH_2\gamma$), 1.59–1.73 (m, 1H, CHβ), 3.80 (d, $J = 15.89$ Hz, 1H, $CH_2\gamma_2$), 3.91 (d, $J = 16.19$ Hz, 1H, $CH_2\alpha$), 4.11 (dd, $J = 6.59$ Hz and 9.00 Hz, 1H, CHα'), 6.21 (s, 2H, $CH_2$2), 7.03 (s, 1H, NH_2-13), 7.05 (s, 1H, Ar-7), 7.35 (s, 1H, NH_2-13), 7.62 (s, 1H, Ar-4), 7.98 (d, $J = 9.29$ Hz, 1H, NH-10).
4e	2.80 (dd, $J = 13.80$ Hz and $J = 4.50$ Hz, 1H, $CH_2\beta$), 3.01 (dd, $J = 13.80$ Hz and 4.80 Hz, 1H, $CH_2\beta'$), 3.73 (d, $J = 16.20$ Hz, 1H, $CH_2\alpha$), 3.82 (d, $J = 16.20$ Hz, 1H, $CH_2\alpha$), 4.43–4.41 (m, 1H, $CH_2\alpha'$), 6.20 (s, 2H, $CH_2$2), 6.88 (s, 1H, Ar-7), 7.0 (s, 1H, NH_2-13), 7.21–7.29 (1H of NH_2-13, 5H of Ar-*m*, *p* and *o*), 8.07 (d, $J = 7.80$ Hz, 1H, NH-10).
4f	2.85 (dd, $J = 9.10$ Hz and 14.10 Hz, 1H, $CH_2\beta$), 3.06 (dd, $J = 5.10$ Hz and 14.10 Hz, 1H, $CH_2\beta'$), 3.35–3.34 (m, 2H, $CH_2\alpha$), 4.61–4.54 (m, 1H, CHα'), 6.77 (s, 1H, Ar-4), 6.97 (d, $J = 8.70$ Hz, 2H, Ar, H-11′ and H-13′), 7.09 (s, 1H, NH_2-13), 7.22 (d, $J = 8.70$ Hz, 2H, Ar-, H-12′ and H-14′), 7.42 (s, 1H, NH_2-13), 7.53 (s, 1H, Ar-7), 7.45–7.56 (m, 3H, Ar), 8.60 (s, 1H, NH-16).
4g	0.76–0.91 (m, 2H, CHγ), 1.10 (s, 9H, $(CH_3)_3$), 1.15–1.28 (m, 2H, $CH_2\gamma'$), 1.68–1.98 (m, 2H, CH_2–NH-11), 4.12 (t, $J = 8.30$ Hz, 1H, NH-11), 4.21 (s, 2H, $CH_2\beta$), 4.63–4.68 (m, 1H, CHα), 6.27 (s, 2H, $CH_2$2), 7.18 (s, 1H, NH_2-14), 7.34 (s, 1H, NH_2-14), 7.44–7.48 (m, 1H, Ar-4), 7.61 (s, 1H, Ar-7), 8.50 (d, $J = 8.39$ Hz, 1H, NH-ε).
4h	1.12 (d, $J = 6.8$ Hz, 3H, $CH_3\Delta$), 1.31–1.60 (m, 1H, CHΔ), 1.76–1.93 (m, 2H, $CH_2\gamma$), 4.12 (s, 1H, $CH_2\gamma'$), 4.24 (dd, $J = 3.9$ Hz and 7.79 Hz, 1H, CHγ'), 6.27 (s, 2H, $CH_2$2), 7.03 (d, 1H, $J = 7.5$ Hz, Ar-6), 7.15 (s, 1H, NH_2-13), 7.21 (d, $J = 7.50$ Hz, 1H, Ar-6), 7.30 (d, 1H, NH_2-13), 7.41 (d, $J = 1.50$ Hz, 1H, Ar-4), 7.57 (d, $J = 7.50$ Hz, 1H, Ar-7), 8.47 (d, $J = 8.3$ Hz, 1H, NH-10).

In general, the antimitotic compounds assayed in the sea urchin eggs presented a higher IC_{50} than that observed for tumour cell lines [35]. However, Safrole was the most active compound in the sea urchin egg's model, while it was only weakly active against tumour cell lines. Probably, the structural requirements for cytotoxic action will depend upon the model used.

2.4. Hemolytic activity

In order to verify whether the observed cytotoxic and antimitotic activities are related to membrane disruption, compounds were tested for their ability to induce lysis of mouse erythrocytes. The erythrocyte membrane is a dynamic structure that can dictate significant changes in its interaction with drugs [36]. The results revealed that only Safrole showed hemolytic activity at the highest tested concentration (200 μg/mL), suggesting that both cytotoxic and antimitotic activities were not related to the lytic properties or membrane instability induced by the Safrole derivatives, which may be probably caused by a more specific pathway.

3. Conclusion

In summary, new peptidyl-like derivatives containing the 1,3-benzodioxole system were synthesized using accessible methodologies. Some derivatives exhibit significant in vitro activity against cancer cell lines, particularly compounds **4f** and **4g**. The cytotoxic effect of the peptidyl-like derivatives can be associated with the DNA-binding in tumour cells, as it is told for Safrole, however, with potentialization of antiproliferative action and selectivity for the phase specifies cellular division.

4. Materials and methods

4.1. Chemistry

All melting points were determined using a Thomas Hoover apparatus and are uncorrected. FTIR spectra were obtained on Brukker spectrophotometer, model IFS66 using KBr pellets. ^{1}H NMR spectra were measured using a Varian UNITY-plus-300 MHz NMR spectrophotometer using DMSO-d_6 as solvent and tetramethylsilane as an internal standard. Elemental analyses were performed on a PE-2400 instrument and the results were in acceptable range. Thin layer chromatography (TLC) was carried out on silica gel plates with a fluorescence indicator of F_{254} (0.2 mm, E. Merck); the spots were visualized in UV light, and by spraying a 2% ethanol solution of ninhydrin or charing reagent. Column chromatography was performed on silica gel using Kiesegel 60 (230–400 Mesh, E. Merck). All reagents used in the present study were of analytical grade.

4.1.1. 5-Allyl-6-nitro-benzo[1,3]dioxole (**2**)

To a stirred mixture of 0.062 mol of 5-allyl-benzo[1,3]dioxole and 0.062 mol of acetic acid under 5 °C, 0.062 mol of concentrated nitric acid in 1.5 mL of acetic acid was slowly added. After 2 h, the mixture was taken up in 100 mL of water and then extracted with three 50 mL portions of ethyl acetate. The organic phase was washed with water, filtered and dried (Na_2SO_4). The residue was chromatographed on silica gel with 2% ethyl acetate in *n*-hexane to give 8.9 g (70%) as a yellow oil.

Table 2
Physical and spectral data for **4a–g**

Compound	Molecular formula[a]	M.p. (°C)[b]	R_f[c]	Yield (%)	IR (KBr) ν (cm^{-1})
4a	$C_{13}H_{15}NO_5$	130–133	0.83	85	3324 *N*N–H, 1628 *N*C=O amide, 1739 *N*C=O ester, 1020 *N*C–O–C.
4b	$C_{12}H_{14}N_2O_4$	135–136	0.85	80	3381 *N*N–H amide 2nd, 1729 *N*C=O assym, 1626 *N*C=O sym, 1503 *N*C=O amide 1st, 1073 *N*C–O–C.
4c	$C_{14}H_{18}N_2O_4$	125–126	0.35	75	3315 *N*N–H amide 2nd, 3405 *N*NH_2 assym, 3347 *N*NH_2 sym, 1689 *N*C=O assym, 1626 *N*C=O sym, 1503 *N*C=O amide 1st, 1073 *N*C–O–C.
4d	$C_{15}H_{19}N_3O_6$	175–176	0.30	70	3326 *N*N–H amide 2nd, 1629 *N*C=O amide, 1574 *N*Ar–NO_2 assym, 1244 *N*Ar–NO_2 sym, 1050 *N*C–O–C.
4e	$C_{18}H_{17}N_3O_6$	158–160	0.40	76	3320 *N*N–H amide 2nd, 1627 *N*C=O amide 1st, 1527 *N*Ar–NO_2 assym, 1330 *N*Ar–NO_2 sym, 1058 *N*C–O–C.
4f	$C_{24}H_{19}N_3O_7Cl_2$	150–153	0.40	46	3326 *N*N–H amide 2nd, 3326 *N*N–H assym, 3193 *N*N–H sym, 1628 *N*C=O amide 2nd, 1532 *N*Ar–NO_2 assym, 1243 *N*Ar–NO_2 sym.
4g	$C_{20}H_{28}N_4O_8$	160–162	0.60	60	3382 *N*N–H of amide 2nd, 1740 *N*C=O amide 1st, 1691 *N*C=O assym, 1658 *N*C=O sym, 1368 *N*Ar–NO_2, 1041 *N*C–O–C.
4h	$C_{15}H_{20}N_2O_4$	153–154	0.70	80	3325 *N*NH amide 2nd, 3380 *N*NH_2 assym, 3323 *N*NH_2 sym, 1672 *N*C=O assym, 1626 *N*C=O sym, 1088 *N*C–O–C.

[a] Microanalysis for the synthesized compounds; Anal. Calc./Found: Compound **4a**: C, 58.86; H, 5.70; N, 5.28/C, 58.01; H, 5.15; N, 4.95. Compound **4b**: C, 57.59; H, 5.64; N, 11.19/C, 57.021; H, 5.11; N, 10.86. Compound **4c**: C, 60.42; H, 6.52; N, 10.07/C, 60.01; H, 6.15; N, 9.86. Compound **4d**: C, 53.41; H, 5.68; N, 12.46/C, 53.00; H, 5.15; N, 12.05. Compound **4e**: C, 58.22; H, 4.61; N, 11.31/C, 58.25; H, 4.65; N, 11.50. Compound **4f**: C, 54.15; H, 3.60; N, 7.98/C, 54.54; H, 3.85; N, 7.82. Compound **4g**: C, 53.09; H, 6.24; N, 12.38/C, 52.89; H, 6.59; N, 12.00. Compound **4h**: C, 61.63; H, 6.90; N, 9.59/C, 61.30; H, 7.10; N, 10.02.
[b] Crystallized from ethyl acetate/*n*-hexane (1:2).
[c] Solvent system ethyl acetate/*n*-hexane (8:2).

*4.1.2. (6-Nitro-benzo[1,3]dioxole-5-yl)acetic acid or (6-H-benzo[1,3]dioxole-5-yl) acetic acid (**3a–b**)*

The aqueous $KMnO_4$ (0.135 mol) in about 150 mL of water was stirred and cooled in an ice bath. A solution of Safrole or compound **2** (0.024 mol), benzyldimethyltetradecylammonium chloride dihydrate (0.0012 mol), 30 mL of acetone, 150 mL of benzene, and 30 mL of acetic acid was added in one portion. After 2 h, stirring continued without any further addition of ice to the bath for 70 h. A total of 40 g of $NaHSO_3$ was added to the cooled reaction mixture followed by the slow addition of 70 mL of aqueous H_2SO_4 (25 g of concentrated H_2SO_4 in 100 mL of water). Two clear layers resulted. The layers were separated and the organic layer was washed once with a 100 mL portion of water. The organic layer was dried over anhydrous sodium sulfate, the drying agent was

Table 3
Cytotoxic activity of the peptidyl-like derivatives on human tumour cell lines[a]

Compound	Entry[b]	HL-60	HCT-8	MDA-MB 435	SF-295
Doxorubicin	–	0.03	0.03	0.83	0.42
1	–	104.5 (84.0–128.4)	36.1 (31.5–41.3)	104.7 (72.8–150)	32.6 (4.1–6.6)
4a	Gly	18.7 (14.4–24)	4.3 (2.5–7.9)	>50	13.5 (9.6–18.5)
4b	Ala	>50	>50	>50	>50
4c	Val	>50	>50	>50	>50
4d	Ileu	>50	>50	>50	19.1 (10.4–34.2)
4e	Phe	>50	>50	>50	>50
4f	Tyr	>50	8.5 (4.3–9.5)	9.9 (8.6–11.4)	>50
4g	Lys	5.4 (3.0–8.5)	>50	9.2 (8.5–11.3)	31.6 (27.2–36.4)
4h	Leu	>50	>50	>50	>50

[a] Data are presented as IC_{50} (μM) values and 95% confidence interval obtained from at least three independent experiments and standard deviation is given in parentheses.
[b] Entry represents the amino acid residue of the compounds.

Table 4
Inhibition of the cell cycle by the peptidyl-like derivatives **4a–h** on the embryos of the sea urchin *Lytechinus variegatus* on the first and third cleavage and blastulae stages

Compound	Concentration (μg/mL)	Sea urchin egg development[a] (%) (mean ± S.E.M.)		
		First cleavage	Third cleavage	Blastulae
Negative control	–	89.3 ± 2.8	76.3 ± 1.1	81.0 ± 3.5
Doxorubicin	10	36.0 ± 1.7*	0.0 ± 0.0*	0.3 ± 0.3*
	100	16.3 ± 2.4*	1.5 ± 1.5*	0.0 ± 0.0*
1	10	59.5 ± 6.4*	33.7 ± 3.5*	31.7 ± 9.3*
	100	0.7 ± 1.2*	0.0 ± 0.0*	0.3 ± 0.6*
4a	10	83.0 ± 10.4	71.3 ± 11.6	83.7 ± 3.2
	100	79.7 ± 6.8	15.7 ± 3.2*	0.3 ± 0.6*
4b	10	76.3 ± 7.5	68.3 ± 2.9	92.0 ± 2.0
	100	76.5 ± 9.2	78.7 ± 2.3	87.7 ± 4.9
4c	10	88.0 ± 1.0	67.0 ± 3.6	96.7 ± 2.3
	100	70.7 ± 2.3*	61.7 ± 2.9	1.0 ± 1.7*
4d	10	88.3 ± 6.0	77.0 ± 5.3	80.3 ± 4.2
	100	94.3 ± 1.2	72.7 ± 4.7	89.0 ± 1.7
4e	10	86.3 ± 4.5	73.3 ± 15.5	92.0 ± 1.0
	100	1.7 ± 2.1*	5.3 ± 4.6*	0.0 ± 0.0*
4f	10	92.3 ± 4.5	38.0 ± 4.0*	1.7 ± 1.5*
	100	85.3 ± 1.5	46.0 ± 2.6*	15.0 ± 2.8*
4g	10	88.7 ± 3.5	72.3 ± 11.6	79.7 ± 4.5
	100	88.7 ± 4.0	66.3 ± 12.9	3.7 ± 3.2*
4h	10	89.3 ± 3.5	62.0 ± 3.5	94.7 ± 2.3
	100	87.7 ± 6.1	70.3 ± 9.3	84.7 ± 7.5

*$p < 0.05$, ANOVA followed by Dunnett's Multiple Comparison test compared to negative control.
[a] The compounds were added 2 min after fertilization of the eggs.

removed by filtration, and the solvent was removed on a rotary evaporator. The crude product was chromatographed on silica gel with 5% ethyl acetate in *n*-hexane to give 3.25 g (60%), as a yellow solid with m.p. 180–182 °C (**3a**) and 3.15 g (58%) as well crystal with m.p. of 192 °C (**3b**).

*4.1.3. General procedure for the synthesis of peptidyl derivatives of the Safrole (**4a–h**)*

To a stirred solution of 2.5 mmol of the TFA–amino acylamide in *N*,*N*-dimethylformamide (20 mL) under 0 °C, 6.8 mmol of the dicyclohexylcarbodiimide, 5.1 mmol of 1-hydroxybenzotriazole and 1.7 mmol of compound **3a** or **3b** were added and was allowed to warm at room temperature. After that, the reaction mixture was filtered, and the filtrate was treated with ethyl acetate. The organic phase was washed with the $NaHCO_3$ solution (50 mL), 1 M citric acid (50 mL), aqueous NaCl (25 mL) and dried (Na_2SO_4) and concentrated. The residue was treated with *n*-hexane and filtered. The derivatives **4a–h** were obtained as yellow crystals.

4.2. MTT assay

The cytotoxicity of the compounds was tested against four tumour cell lines: SF-295 (human glyoblastoma), HCT-8 (human colon carcinoma), HL-60 (human myeloblastic leukemia) and MDA/MB-435 (human breast) (National Cancer Institute, USA). Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C with 5% CO_2. For experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.3×10^6 cells/well for suspended cells in 100 μL medium). After 24 h, the compounds (0.39–25 μg/mL) dissolved in DMSO (5%) were added to each well and incubated for 3 days (72 h). Control groups received the same amount of DMSO. Doxorubicin was used as positive control. Growth of tumoural cells was quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product. At the end of 72 h incubation, the medium in each well was replaced by fresh medium (200 μL) containing 0.5 mg/mL of MTT. Three hours later, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multi-plate reader (Spectra Count, Packard, Ont., Canada). Drug effect was quantified as the percentage of control absorbance of reduced dye at 590 nm.

4.3. Assay on sea urchins

The test was performed in 24-well plates following the method described by Costa-Lotufo et al. [35]. Adult sea urchins (*Lytechinus variegatus*) were collected at Pecém beach, on the northeastern coast of Brazil. The gamete elimination was induced by injecting 3.0 mL of 0.5 M KCl into the urchin's coelomic cavity via the periostomial membrane. The eggs were washed twice using filtered seawater to remove the jelly coat surrounding the cells. Concentrated sperm was collected with a Pasteur pipette and maintained under low temperature until the moment of fertilization. For fertilization, 1 mL of a sperm suspension (0.05 mL of concentrated sperm in 2.45 mL of filtered seawater) was added to every 50 mL of egg solution. Each well received 1 mL of fertilized egg suspension. The compounds were added immediately after fertilization (within 2 min) to get concentrations of 10 and 100 μg/mL in a final volume of 2 mL. Doxorubicin was used as positive control. The plates were then shaken in a constant temperature water bath at 26 ± 2 °C. At appropriate intervals, aliquots of 200 μL were fixed with the same volume of 10% formaldehyde to obtain first and third cleavages, and blastulae. One hundred eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells.

4.4. Hemolytic assay

The test was performed in 96-well plates following the method described by Costa-Lotufo et al. [35]. Each well received 100 μL of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well was the negative control that contained only the vehicle (distilled water or DMSO 10%), and, in the second well, 100 μL of test substance that was diluted to half was added. The compounds were tested at concentrations ranging from 10 to 200 μg/mL. The serial dilution continued until the eleventh well. The last well received 20 μL of 0.1% Triton X-100 (in 0.85% saline) to obtain 100% hemolysis (positive control). Then, each well received 100 μL of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm.

4.5. Statistical analysis

The IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). For the sea urchin egg's assay, the differences were analyzed by ANOVA followed by Dunnett's Multiple Comparison test compared to negative control at a significance level of 5%.

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