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Design, synthesis and anti-trypanosomatid activities of 3,5-diaryl-isoxazole analogues based on neolignans veraguensin, grandisin and machilin G

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Abstract

Using bioisosterism as a medicinal chemistry tool, 16 3,5-diaryl-isoxazole analogues of the tetrahydrofuran neolignans veraguensin, grandisin and machilin G, were synthesized via 1,3-dipolar cycloaddition reactions, with yields from 43–90%. Anti-trypanosomatid activities were evaluated against *Trypanosoma cruzi*, *Leishmania (L.) amazonensis* and *Leishmania (V.) braziliensis*. All compounds were selective for the *Leishmania* genus and inactive against *T. cruzi*. Isoxazole analogues showed a standard activity on both promastigotes of *L. amazonensis* and *L. braziliensis.* The most active compounds were **15**, **16** and **19** with IC_{50} values of 2.0, 3.3 and 9.5 µM against *L. amazonensis* and IC_{50} values of 1.2, 2.1 and 6.4 µM on *L. braziliensis*, respectively. All compounds were non-cytotoxic, showing lower cytotoxicity (>250 µM) than pentamidine (78.9 µM). Regarding the structure-activity relationship (SAR), the methylenedioxy group was essential to antileishmanial activity

against promastigotes. Replacement of the tetrahydrofuran nucleus by an isoxazole core improved the antileishmanial activity.

Keywords: Neolignans, cycloaddition [3+2], isoxazole, bioisosterism.

1. Introduction

Leishmaniasis is among many neglected diseases prevailing in underdeveloped tropical countries in Asia, Africa and Central America and South America.[1] Ranking just after malaria and with over one billion people worldwide at risk of infection, leishmaniasis is a severe health problem. The main clinical forms of the disease are cutaneous, mucocutaneous and visceral.[1,2]

The first-line treatment for leishmaniasis consists of administration of pentavalent antimonials such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®). However, the use of antimonial therapy is limited due to its hepatotoxicity, cardiotoxicity, and nephrotoxicity, as well as other diverse collateral effects.[3]

When these drugs are ineffective or cannot be prescribed for other reasons, the second line of treatment typically involves amphotericin B, pentamidine or paromomycin.

However, the adverse side effects and parasite resistance to all of these drugs underscore the urgency of developing newer and safer therapeutic agents for the treatment of leishmaniasis, as well as other neglected diseases.[4]

Chagas disease, caused by *Trypanosoma cruzi*, is another member of the neglected-disease family. With a high mortality and morbidity rate, affecting over six million people in Latin American countries, its treatment is restricted to two drugs,

benznidazole, and nifurtimox, which are only useful during the acute phase of the disease.[5]

Benznidazole is the first-choice drug in most cases, even though it presents problems such as a narrow therapeutic window, side effects and different susceptibility of *T. cruzi* strains.[6]

Currently available treatments for neglected diseases are far from ideal. It is therefore, imperative to emphasize the need for continuous research on the development of new and useful candidate drugs.

Some outstanding drug-discovery programs are widely used to find leading compounds, based on and inspired by the molecular modifications of natural products. Recent studies have demonstrated the potential of this approach for the discovery of new chemical entities in the treatment of neglected diseases.[2,6–10]

Tetrahydrofuran neolignans, derived from natural sources are considered privileged structures since they show several biological activities including antibacterial, antifungal, anti-tumour, and anti-inflammatory. The tetrahydrofuran neolignans veraguensin (**1)**, grandisin (**2)**, and machilin G (**3)**, for instance (Figure 1), have shown anti-trypanosomal and anti-leishmanial activities (Figure 1).[11–21]

Regarding the biological activities of compounds **1-3**, IC_{50} values were described of 2.3, 3.7 and 2.2 μM, respectively, against *T. cruzi*.[18-20] Veraguensin (**1)** and machilin G (**3)** showed an IC_{50} value of 18 μg mL^{-1} (48.8 and 50.54 μM respectively) against *L. donovani*.[13] These results indicate that neolignans **1-3** are more active against *T. cruzi* than the leishmaniasis.

Considering that our research group is interested in developing new ligands against leishmaniasis and Chagas disease we developed compounds via molecular modification of neolignans **1–3**, using a bioisosterism approach to obtain analogues with increased potency. Thus, the tetrahydrofuran core from the neolignans was replaced by a 1,2,3-triazole moiety (Figure 2).

The position isomers of 1,2,3-triazole analogues showed diverse antileishmanial activities. The triazole hybrid **4**, from grandisin **2** and machilin G **3** was the most active against the promastigotes and amastigotes of *Leishmania (L.) amazonensis* (Figure 2).[2,8]

In order to increase the compound series based on the tetrahydrofuran neolignan family, our research group is particularly interested in the isoxazole core. Several compounds containing this nucleus have shown interesting biological activities including antimicrobial, antiviral, anticancer, anti-inflammatory, immunomodulatory, anticonvulsant, anti-diabetic and anti-trypanosomatid.[22] The isoxazole core is a bioisostere of some heterocyclic rings and was used in the design of valdecoxib.[23] It shows a structural resemblance to the 1,2,3-triazole nucleus, and is also a hydrogen-bond acceptor.[24,25]

Therefore, we theorized that the isoxazole core must be a ring equivalent and bioisostere of the tetrahydrofuran nucleus and the 1,2,3-triazole ring. In this way, the aim of this study was to obtain 16 new, synthetic isoxazole hits inspired by neolignan structures **1–3** and to evaluate their anti-trypanosomatid activities (Figure 2).

2. Experimental

2.1. General remarks

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on Brucker 75 MHz or 300 MHz spectrometer, with deuterated chloroform solution ($CDCl_3$) or $DMSOd_6$. For NMR data, chemical shifts (δ) are expressed as parts per million (ppm) downfield with values related to tetramethylsilane (TMS) used as an internal standard. High-resolution electrospray ionization (HR-ESI-MS) measurements were carried out on a quadrupole time-of-flight instrument (UltrOTOF-Q, Bruker Daltonics, Billerica, MA). Infrared spectrums (IR) were recorded on a Thermo Scientific Nicolet iS5® system equipped with an iD3 attenuated total reflectance (ATR) attachment (germanium crystal). All solvents were dried and distilled before use according to the standard procedure. All reactions were performed under an atmosphere of dry nitrogen and monitored by thin layer chromatography (TLC) using prepared plates (silica gel 60 F254 on aluminium). Flash column chromatography was performed using silica gel 60 (particle size 200-400 mesh ASTM, purchased from Sigma-Aldrich, (USA). Melting points (mp) were determined using a Fisatom 430D. Terminal acetylenes, oximes, and chloro-oximes were synthesized using procedures from the literature.[2,8,26,33-39] The spectra data for all isoxazole analogs **5-20** as, ^{1}H NMR, ^{13}C NMR, IR and High-Resolution Mass Spectrometry, is provided in the Supporting Information.

2.2. Synthesis

General procedure for the preparation of Isoxazole compounds 5-16[26]

To a solution of the respective chloro-oxime **29a-c** (2 mmol) and the acetylenes **26a-d** (2.1mmol) in CH_2Cl_2/THF 1:1, 4 mL/mmol, $CuSO_4.5H_2O$ (0.15 mmol), sodium ascorbate (0.35 mmol) and $KHCO_3$ (20 mmol) were added. The mixture was stirred at room temperature for 48 hours. Extraction was performed with ethyl acetate and washed with a saturated solution of NH_4Cl (3 x 30 mL), the organic phase was dried over anhydrous $MgSO_4$, and solvent was removed under vacuum.

3,5-bis-(4-methoxyphenyl) isoxazole 5.[40] The product was purified by crystallization in heated hexane/ethyl acetate solution (90:10). The product was obtained as a yellow solid, mp = 160 °C. Yield: 90%.^{1}H NMR (300 MHz, $CDCl_3$) δ 3.84 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 6.63 (s, 1H, CH); 6.97 (d, J = 8.7 Hz, 4H, Ar-H); 7.75 (d, J = 8.7 Hz, 2H, Ar-H); 7.78 (d, J = 8.7 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.3; 55.4; 95.91; 114.3; 114.4; 120.4; 121.8; 127.4; 128.2; 160.9; 161.1; IR (ATR): 2842; 2922; 2851; 1575; 1502; 1436; 1260; 1123; 1123; 1021; 860; 841; 804 v_{max}(cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{17}H_{15}NO_3$ [M + H]: 282.1124; found: 282.1134.

3,5-bis-(3,4-dimethoxyphenyl) isoxazole 6. The product was purified by crystallization in a heated hexane/ethyl acetate solution (90:10). The product was obtained as a white solid, mp = 131 °C. Yield: 55%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.92 (s, 6H, 2xOCH_3); 3.95 (s, 6H, 2xOCH_3); 6.66 (s, 1H, CH); 6.93 (dd, J = 9.0 and 3.0 Hz, 2H, Ar-H); 7.53 (d, J = 1.5 Hz, 1H, Ar-H); 7.66 (m, 2H, Ar-H); 8.03 (s, 1H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.9; 56.0; 56.0; 56.0; 96.2; 108.7; 109.4; 111.1; 111.3; 119.1; 119.9; 120.5; 122.0; 142.3; 150.6; 150.7; 162.7; 170.2. IR (ATR): 1608.; 1509; 1435; 1269.90; 1222; 1143; 1020; 862; 800; 765 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{19}H_{19}NO_5$ [M + H]: 342.1359; found: 342.1353.

3,5-bis-(1,3-benzodioxol-5-yl) isoxazole 7. The product was purified by crystallization in a heated hexane/ethyl acetate (90:10) solution. The product was obtained as a yellow solid, mp =193°C, Yield: 75%.^{1}H NMR (300 MHz, $CDCl_3$) δ 6.01 (s, 2H, CH_2); 6.02 (s, 2H, CH_2); 6.58 (s, 1H, CH); 6.86 (d, J = 8.1 Hz, 1H, Ar-H); 6.87 (d, J = 8.1 Hz, 1H, Ar-H); 7.37 (dd, J = 8.3 and 1.9 Hz, 1H, Ar-H); 8.02 (d, J = 9.1 Hz, 2H, Ar-H); 8.42 (d, J = 8.9 Hz, 2H, Ar-H).^{13}C NMR ($CDCl_3$, 75 MHz) δ 96.4; 101.4; 101.6; 106.1; 106.96; 108.6; 108.8; 120.4; 121.1; 121.6; 123.1; 148.2; 148.2; 149.1; 149.3; 162.5; 170.0 IR (ATR): 2921; 2851; 1592;

1499; 1459.; 1253; 1038; 866; 817; 795 $v_{max}(cm^{-1})$. HRMS (ESI+) *m/z* calcd for $C_{17}H_{11}NO_5$ [M + H]: 310.0709; found: 310.0705.

5-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl) isoxazole 8. The product was purified by crystallization in a heated hexane/ethyl acetate solution (90:10). The product was obtained as an off white solid, mp= 111°C. Yield: 72%.^{1}H NMR (300 MHz, $CDCl_3$) δ 3.85 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 3.95 (s, 3H, OCH_3); 6.66 (s, 1H, CH); 6.93 (d, *J* = 8.4 Hz, 1H, Ar-H); 6.97 (d, *J* = 8.7 Hz, 2H, Ar-H); 7.32 (d, *J* = 1.8 Hz, 1H, Ar-H); 7.39 (dd, *J* = 8.1 and 1.8 Hz, 1H, Ar-H); 7.78 (d, *J* = 8.7 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.4; 56.0; 56.1; 96.2; 108.7; 111.3; 114.3; 119.1; 120.5; 121.7; 128.2; 149.3; 150.7; 162.0; 162.6; 170.1. IR (ATR): 2998; 2840; 1616; 1509; 1441; 1247; 1178; 1141; 861; 791 $v_{max}(cm^{-1})$. HRMS (ESI+) *m/z* calcd for $C_{18}H_{17}NO_4$ [M + H]: 312,1236; found: 312.1239.

3-(3,4-dimethoxyphenyl)-5-(4-methoxyphenyl) isoxazole 9. The product was purified by crystallization in a heated hexane/ethyl acetate solution (90:10). The product was an off white solid, mp = 121 °C. Yield: 55%.^{1}H NMR (300 MHz, $CDCl_3$) δ 3.85 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 3.95 (s, 3H, OCH_3); 6.66 (s, 1H); 6.92 (d, *J* = 9.0 Hz, 1H); 6.97 (d, *J* = 9.0 Hz, 2H); 7.33 (dd, *J* = 9.0 and 2.2 Hz, 1H); 7.45 (d, *J* = 2.1 Hz, 1H); 7.76 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.4; 56.0; 56.0; 96.0; 109.3; 111.0; 114.4; 119.9; 120.4; 122.0; 127.4; 149.3; 150.53; 161.1; 170.2. IR (ATR): 1616; 1432; 1251; 1172; 1144; 1024; 832; 809 $v_{max}(cm^{-1})$. HRMS (ESI+) *m/z*calcd for $C_{18}H_{17}NO_4$ [M + H]: 312.1230; found: 312.1230.

5-(1,3-benzodioxol-5-yl)-3-(4-methoxyphenyl) isoxazole 10. The product was purified by crystallization in a heated hexane/ethyl acetate solution (90:10). The product was obtained as an off white solid, mp= 137°C. Yield: 68%.^{1}H NMR (300 MHz, $CDCl_3$) δ 3.84 (s, 3H,

OCH_3); 6.02 (s, 2H, CH_2); 6.61 (s, 1H, CH); 6.88 (d, *J* = 8.1 Hz, 1H, Ar-H); 6.97 (d, *J* = 9.0 Hz, 2H, Ar-H); 7.26 (d, *J* = 1.5 Hz, 1H, Ar-H); 7.34 (dd, *J* = 8.1 and 1.5 Hz, 1H, Ar-H); 7.76 (d, *J* = 7.2 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.3; 96.3; 101.6; 106.1; 108.8; 113.8; 114.3; 120.4; 121.7; 121.7; 128.2; 148.2; 149.2; 161.0.; 162.6; 169.9. IR (ATR): 1613; 1502; 1492; 1437; 1392.84; 1250.; 1179; 1109; 825; 804 v_{max} (cm^{-1}). HRMS (ESI+) *m/z*calcd for $C_{17}H_{13}NO_4$ [M + H]: 296.0917; found: 296.0923.

3-(1,3-benzodioxol-5-yl)-5-(4-methoxyphenyl) isoxazole 11. The product was purified by crystallization in a heated hexane/ethyl acetate (90:10) solution. The product obtained was a yellow solid, mp = 162°C, Yield: 77%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.85 (s, 3H, OCH_3); 6.01 (s, 2H, CH_2); 6.60 (s, 1H, CH); 6.88 (d, *J* = 9.0 Hz, 1H, Ar-H); 6.97 (d, *J* = 9.0 Hz, 2H, Ar-H); 7.30 (dd, *J* = 9.0 and 1.5 Hz, 1H, Ar-H); 7.35 (d, *J* = 1.5 Hz, 1H, Ar-H); 7.74 (d, *J* = 6.0 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.4; 96.0; 101.4; 107.0; 108.6; 114.4; 120.3; 121.1; 123.3; 127.4; 148.2; 149.05; 161.1; 162.6; 170.2. IR (ATR): 2921; 2851; 1612; 1504; 1457; 1250; 1180; 1114; 1030; 872; 825; 804 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{17}H_{13}NO_4$ [M + H]: 296,0917; found: 296,0932.

3-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl) isoxazole 12. The product was purified by crystallization in heated a hexane/ethyl acetate solution (90:10). The product was obtained as an off white solid, mp= 156°C. Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.84 (s, 3H, OCH_3); 3.88 (s, 3H, OCH_3); 3.92 (s, 6H, 2xOCH_3); 6.70 (s, 1H, CH); 6.96 (d, *J* 8.7 Hz, 2H, Ar-H); 7.02 (s, 2H, Ar-H); 7.77 (d, *J* 8.7 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.3; 56.3; 61.0; 97.0; 103.2; 114.3; 121.6; 122.9; 128.2; 139.8; 153.6; 161.0; 162.7; 169.9. IR (ATR): 1615; 1571; 1498; 1418; 1185; 1125; 999; 839; 795 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{19}H_{19}NO_5$[M + H]: 342.1335; found: 342.1328.

3-(3,4-dimethoxyphenyl)-5-(3,4,5-trimethoxyphenyl) isoxazole 13. The product was purified by crystallization in a heated hexane/ethyl acetate solution (90:10). The product was obtained as a yellow solid, mp = 107 °C, Yield: 52%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.88 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 3.92 (s, 6H, 2xOCH_3); 3.95 (s, 3H, OCH_3); 6.70 (s, 1H, CH); 6.92 (d, *J* = 8.4 Hz, 1H, Ar-H); 7.02 (s, 2H, Ar-H); 7.34 (dd, *J* = 8.4 and 1.8 Hz, 1H, Ar-H); 7.44 (d, *J* = 1.8 Hz, 1H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.9; 56.0; 56.3; 61.0; 97.0; 103.2; 109.4; 110.0; 112.0; 119.9; 120.0; 120.9; 121.8; 122.9; 139.9; 149.3; 150.6; 153.6; 162.7, 170.0. IR (ATR): 2922; 2851; 1575; 1502; 1436; 1260; 1123; 1021; 860; 841; 804 v_{max}(cm^{-1}). HRMS (ESI+) *m/z*calcd for $C_{20}H_{21}NO_6$ [M + H]: 372,1441; found: 372,1446.

5-(1,3-benzodioxol-5-yl)-3-(3,4-dimethoxyphenyl) isoxazole 14. The product was purified by flash chromatography on silica gel using a hexane/ethyl acetate solution (90:10), as the mobile phase which resulted a white solid, mp = 140 °C, Yield: 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.90 (s, 3H, OCH_3); 3.93 (s, 3H, OCH_3); 6.00 (s, 2H, CH_2); 6.61 (s, 1H, CH); 6.86 (d, *J* = 8.1 Hz, 1H, Ar-H); 6.90 (d, *J* = 8.4 Hz, 1H, Ar-H); 7.23 (d, *J* = 1.2 Hz, 1H, Ar-H); 7.29 (dd, *J* = 8.7 and 1.2 Hz, 1H, Ar-H); 7.32 (dd, *J* = 8.1 and 1.5 Hz, 1H, Ar-H); 7.42 (d, *J* = 1.8 Hz, 1H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.9; 56.0; 96.3; 101.6; 106.1; 108.8; 109.3; 111.0; 119.8; 120.4; 121.6; 121.9; 148.2; 149.2; 149.2; 150.57; 162.6; 169.9. IR (ATR): 2922; 2851; 1607; 1495; 1434.; 1257; 1226; 1173; 1143; 1032; 852; 820; 804 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{18}H_{15}NO_5$ [M + H]: 326.1022; found: 326.1021.

3-(1,3-benzodioxol-5-yl)-5-(3,4-dimethoxyphenyl) isoxazole 15. The product was purified by crystallization in heated hexane/ethyl acetate solution (90:10). The product was obtained as a yellow solid, mp = 133°C, Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.92 (s, 3H, OCH_3); 3.95 (s, 3H, OCH_3); 6.01 (s, 2H, CH_2); 6.62 (s, 1H, CH); 6.93 (d, *J* = 8.1 Hz, 1H, Ar-

H); 6.88 (d, *J* = 8.4 Hz, 1H, Ar-H); 7.30 (dd, *J* = 9.0 and 1.5 Hz, 1H, Ar-H); 7.35 (d, *J* 1.5 Hz, 1H, Ar-H); 7.74 (d, *J* 6.0 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.9; 56.0; 96.2; 101.4; 106.9; 108.5; 111.2; 119.0; 120.3; 121.0; 123.1; 148.1; 149.0; 149.2; 150.6; 162.5; 170.1. IR (ATR): 2922; 2851; 1607; 1508; 1465; 1252; 1139; 1035; 818; 809; 797 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{18}H_{15}NO_5$ [M+H]: 326,1022; found: 326,1032.

3-(1,3-benzodioxol-5-yl)-5-(3,4,5-trimethoxyphenyl) isoxazole 16. The product was purified by crystallization by heated in hexane/ethyl acetate solution (90:10). The product was obtained as a yellow solid, mp = 138 °C, Yield: 71%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.88 (s, 3H, OCH_3); 3.91 (s, 6H, 2xOCH_3); 6.01 (s, 2H, CH); 6.66 (s, 1H, CH_2); 6.87 (d, *J* = 8.1 Hz, 1H, Ar-H); 7.01 (s, 2H, Ar-H); 7.30 (dd, *J* = 8.1 and 1.5 Hz, 1H, Ar-H); 7.35 (d, *J* = 1.5 Hz, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 56.3; 61.0; 97.0; 101.5; 103.1; 107.0; 108.6; 121.1; 122.8; 123.0; 139.8; 148.2; 149.2; 153.6; 162.6; 170.0. IR (ATR): 2922; 2851; 1574; 1503; 1464; 1421; 1248; 1125; 1039; 875; 847.08; 823; 807 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{19}H_{17}NO_6$ [M + H]: 356.1128; found: 356.1128.

General procedure for the preparation of isoxazole compounds 17-20.[32]

A mixture of the *N*-chlorosuccinimide (2 mmol), pyridine (4 drops), and oxime **28d** (2 mmol) in $CHCl_3$, was stirred by 12 h at room temperature. After terminal acetylenes **26a-d** (2.1 mmol), followed by triethylamine (4mmol) were added. The mixture was stirred at room temperature for 48 hours. Extraction was performed with ethyl acetate and washed with a saturated solution of NH_4Cl (3 x 50 mL). The organic phase was dried over anhydrous $MgSO_4$, and solvent was removed under vacuum.

3-(3,4,5-trimethoxyphenyl)-5-(4-methoxyphenyl) isoxazole 17. The product was purified by flash chromatography on silica gel using a hexane/ethyl acetate (90:10) solution as the mobile phase. The product was obtained as an off-white solid, mp = 168°C, Yield: 49%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.88 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 3.96 (s, 6H, 2xOCH_3); 6.69 (s, 1H, CH); 7.01 (d, *J* = 6.0 Hz, 2H, Ar-H); 7.09 (s, 2H, Ar-H); 7.79 (d, *J* = 6.0 Hz, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.4; 56.3; 60.9; 96.1; 104.1; 114.4; 120.2; 124.7; 127.4; 153.6; 161.2; 162.8; 170.5. IR (ATR): 2965; 2937; 1615; 1505; 1431; 1235; 1125; 1000; 841; 794 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{19}H_{19}NO_5$ [M + H]: 342.1341; found: 342.1359.

3-(3,4,5-trimethoxyphenyl)-5-(3,4-dimethoxyphenyl) isoxazole 18. The product was purified by flash chromatography on silica gel using a hexane/ethyl acetate (90:10) solution as the mobile phase. The product was obtained as a yellow solid, mp = 118°C. Yield: 51%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.91 (s, 3H, OCH_3); 3.96 (s, 9H, 3xOCH_3); 3.99 (s, 3H, OCH_3); 6.70 (s, 1H, CH); 6.96 (d, *J* = 9.0 Hz, 1H, Ar-H); 7.09 (s, 2H, Ar-H); 7.35 (d, *J* = 1.0 Hz, 1H, Ar-H); 7.42 (dd, *J* = 9.0 and 1.0 Hz, 1H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 56.0; 56.1; 56.3; 61.0; 96.4; 104.1; 108.7; 111.3; 119.2; 120.37; 124.6; 149.3; 150.8; 153.6; 162.9; 170.4. IR (ATR): 2937; 2837; 1611; 1505; 1424; 1269.41; 1236; 1131; 1022; 858; 849; 786 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{20}H_{21}NO_6$ [M + H]: 372.1447; found: 372.1441.

5-(1,3-benzodioxol-5-yl)-3-(3,4,5-trimethoxyphenyl) isoxazole 19. The product was purified by flash chromatography on silica gel using a hexane/ethyl acetate (90:10) solution as the mobile phase. The product was obtained as a white solid, mp= 142 °C. Yield: 45%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.87 (s, 3H, OCH_3); 3.90 (s, 6H, 2xOCH_3); 6.00 (s, 2H, CH_2); 6.62 (s, 1H, CH); 6.86 (d, *J* = 9.0 Hz, 1H, Ar-H); 7.02 (s, 2H, Ar-H); 7.23 (d, *J* = 3.0 Hz, 1H,

Ar-H); 7.31 (dd, *J* = 9.0 and 3.0 Hz, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 56.2; 60.9; 96.5; 101.6; 104.0; 106.1; 108.8; 120.4; 121.5; 124.5; 139.6; 148.3; 149.3; 153.6; 162.8; 179.2. IR (ATR): 2905; 2853; 1581; 1505; 1493; 1469; 1451; 1423; 1265; 1228; 1112; 1037; 998; 840; 781 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{19}H_{17}NO_6$ [M + H]: 356.1129; found: 356.1134.

3,5-bis-(3,4,5-trimethoxyphenyl) isoxazole 20. The product was purified by flash chromatography on silica gel using a hexane/ethyl acetate (90:10) solution as the mobile phase. The product was obtained as a yellow solid, mp = 143 °C. Yield: 43%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.91 (s, 6H, 2xOCH_3); 3.95 (s, 12H, 4xOCH_3); 6.67 (s, 1H, CH); 7.05 (s, 2H, Ar-H); 7.08 (s, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 26.7; 56.3; 61.0; 97.2; 103.2; 104.1; 122.8; 153.6. IR (ATR): 2925; 2852; 1574; 1502; 1422; 1236; 1125; 999; 854; 833; 778 v_{max} (cm^{-1}). HRMS (ESI+) *m/z* calcd for $C_{21}H_{23}NO_7$ [M + H]: 402.1553; found: 402.1549.

2.2. Biological Methods

2.2.1. Antileishmanial assays

Leishmania (Leishmania) amazonensis

In 96-well microplates, the synthetic compounds **5-20** were tested in quintuplicates, with final concentrations of 0.78 μg/mL to 50 μg/mL. Promastigotes forms of *L. amazonensis* (IFLA/BR/1967/PH8 strain) cultivated in Schneider's Insect Medium(Sigma-Aldrich®) were added to the wells in the exponential phase with a concentration of $2x10^5$ parasites/mL. The plates were incubated at 26°C for 72 h. To evaluate the cell viability, 20 μL (5mg/mL) of Thiazolyl Blue Tetrazolium Bromide - MTT (Sigma-Aldrich®),[41] were added in each well and incubated 37°C, 5% CO_2 for 2h. After that time, 80 μL of DMSO was added to finish the reaction.

Pentamidine (Sigma-Aldrich®) was used as positive control. Dimethyl sulfoxide (DMSO, Sigma-Aldrich®), in Schneider's Insect Medium, was used as negative control and did not interfere with cell viability. The reaction results were obtained in a spectrophotometer (Biotek) at 540 nm. A non-linear dose-response regression curve was used to calculate the half maximum inhibitory concentration (IC_{50}).

The percentage of activity was calculated by the following formula:

% Activity = 100 - [(N-Y) / (N-P)] * 100

where:

Y = optical density reading of cells and wells with different concentrations of the compounds;

N = optical density reading of parasites in wells with 1.5% DMSO;

P = optical density reading of the wells with only culture medium.

Pentamidine (IC_{50}) promastigotes = 8.9 μM

Leishmania (viannia) braziliensis

The compounds **5-20** were tested in triplicates (0.5; 2.0; 8.0, and 32.0 μM) against promastigotes forms of *L. braziliensis,* previously adjusted to $1x10^6$ cells/mL in 96 well plate. The plate was incubated in a humidified atmosphere at 22°C for 72 hours. Subsequently, the viability of the promastigotes was tested by Thiazolyl Blue Tetrazolium Bromide - MTT (Sigma-Aldrich®),[41]which was added to 50mL of an XTT solution containing PMS (phenazine methosulfate), in the proportion of 1 mg/mL of XTT to 0.001 mg/mL of PMS. The plate was then incubated in a humid atmosphere at 37°C and 5% CO_2 for 4 hours, protected from light. At the conclusion of the test, the reaction results were obtained in a

spectrophotometer (Biotek) at 450 nm. The positive control was Schneider's medium and parasites. DMSO 1.5% was used as negative control.

The percentage of activity was calculated by the following formula:

% Activity = 100 - [(N-Y) / (N-P)] * 100

where:

Y = optical density reading of cells and wells with different concentrations of the compounds;

N = optical density reading of parasites in wells with 1.5% DMSO;

P = optical density reading of the wells with only culture medium.

Pentamidine (IC_{50}) promastigotes = 10.1μM

2.2.2. Trypanocidal assay[2]

The assays against trypomastigotes forms of *T. cruzi* were carried out in 96 wells microplates. The trypomastigotes were obtained from the supernatant of cellular culture. The concentration of parasites was adjusted to 10^6 forms/mL in RPMI medium, supplemented by antibiotics and fetal bovine serum. The compounds **5-20** were added at concentrations of 0.5, 2.0, 8.0 and 32.0μM. All the compounds were evaluated in triplicates.

The material was incubated for 24 hours at 4 °C. After this period, to each well was added a 10μL solution of FluoReporter lacZ/Galactosidase quantitative Kit (Life Technologies), and the plates were incubated again for 30 minutes. The colorimetric reaction was quantified by fluorescence microplates reader (BIOTEK), at 386 nm excitation and 448 nm emissions.

The percentages of parasite lysis were determined from the following formula.

% lysis = 100 - {[(X-CP) / (PC-NC)]} .100

where:

X = optical density value of the samples;

CP = optical density value of the positive controls;

CN = optical density value of the negative controls.

Culture medium was used as positive control (CP) and medium with DMSO 0.6% as a negative control.

Benznidazole (IC_{50}): tripomastigotes = 7.32 μM

2.2.4. Cytotoxicity assay[2]

Fibroblasts (NIH/3T3) obtained from Rio de Janeiro Cell Bank (Brazil), were seeded in 96 well plates ($1x10^4$ cells mL^{-1}) and incubated with synthetic compounds at 37°C, 5% CO_2 for 48h at the concentrations of 0.25 to 250 μg/mL. Doxorubicin (Sigma-Aldrich®) was used as the reference drug at concentrations of 0.025 to 25 μg/mL. Cell growth was estimated by the sulforhodamine B colorimetric method (SRB).[42]DMSO (Vetec®) was used as a negative control at the concentration necessary to solubilize the highest concentration of the test samples and did not interfere with cell viability. The percentage of growth was calculated as described by Monks et al.[43] IC_{50} was determined by non-linear regression analysis (Microcal Origin Versão 6.0® e Microsoft Office Excel 2007®). Selectivity index (SI) was calculated according to Medeiros et al.[44]

3. Results and discussion

3.1. Chemistry

The isoxazole compounds were synthesized by a cycloaddition reaction [3+2] between terminal acetylenes and chloro-oximes catalyzed by copper salts.[26] Aryl bromides were

obtained through the reaction of **21a-c** with NBS, p-TsOH, CH_2Cl_2 and SiO_2 (Scheme 1).[2,27] More-reactive aryl iodides can also be used as starting materials.[28]

The cross-coupling reaction between aryl bromines **22a-c** and 2-methyl-3-butyn-2-ol in the presence of $PdCl_2(PPh_3)_2$/CuI, Et_3N, for a duration of 24 h, produced acetylene alcohols **23a-c** with a 75–85% yield (Scheme 1).[2,29]

The Retro-Favorski reaction between **23a-c** with KOH under reflux in toluene generated terminal acetylenes **26a-c** with 65–85% yield.[30] Ethynyl-1,2,3-trimethoxybenzene **26d** was synthesized by the Corey-Fuchs method (Scheme 1).[31]

Oximes were prepared by reaction of methoxy benzaldehydes **27a-c** with $NH_2OH.HCl$/KOH in H_2O/EtOH 1:1 within a yield range of 85–97%. Chloro-oximes **29a-c** were synthesized by reaction between oximes **28a-c** and N-chlorosuccinimide (NCS) in DMF with significant yields (Scheme 2).[26]

The cycloaddition [3+2] reaction was performed between terminal acetylenes **26a-d** and chloro-oximes **29a-c** using a catalytic system of $CuSO_4.5H_2O$, sodium ascorbate, and $KHCO_3$ in a solvent mixture of THF/CH_2Cl_2 1:1 (Table 1).[26]

Additional solvent mixtures were tested as well, and included: CH_2Cl_2/H_2O, THF/H_2O, *t*-BuOH/THF, *t*-BuOH/H_2O, CuI as copper salt and TEA or $NaHCO_3$ as bases. However, the most promising results were achieved using the method described above.

The synthesis of positional isomers derived from trimethoxy oxime **28d** was not successful because this compound was chlorinated in the aromatic nucleus[32a] when NCS in DMF was used, providing **30** with 100% conversion (Scheme 3).

However, when the reaction was performed between trimethoxy oxime **28d** and acetylenes **26a-d**, using NCS, $CHCl_3$, pyridine, and Et_3N at room temperature, in a one-pot procedure, isoxazoles **17-20** were obtained in moderated yields (Scheme 4).[32b-c] Chlorinated isoxazoles were also formed in a minor proportion (15-20%), but the reaction mixture was readily purified by flash chromatography.

All isoxazole compounds **5-20**, synthesized in our study were analysed using nuclear magnetic resonance ^{1}H NMR, ^{13}C NMR, IR and High-Resolution Mass Spectrometry.

3.2. Biological evaluation

Antitrypanosomatid *in vitro* activity were targeted the stationary promastigote forms of *Leishmania (L.) amazonensis* and the trypomastigote forms of *Trypanosoma cruzi* (Table 2).

All synthesized compounds showed limited or no biological activity against *T. cruzi* with IC_{50} values over 150 µM. These results indicate a selectivity of isoxazole analogues for the *Leishmania* genus.

Regarding the antileishmanial activities against *L. amazonensis* strains, we observed that the symmetrical compounds **5**, **6**, **7** and **20**, exhibited different patterns of biological activities. Compound **5** was inactive against *L. amazonensis* strains. Compound **6** showed an IC_{50} of 34 µM. Compound **7**, containing two methylenedioxy groups, had an IC_{50} of 13.9 µM. Compound **23**, a grandisin isoxazole derivative, showed an IC_{50} of 18.3 µM.

Hybrid asymmetrical compounds **8–19** showed a diverse set of biological activities against *L. amazonensis* strains.

Positional isomers such as hybrids **8** and **9**, showed IC_{50} values higher than 160μM. Isomers **10** and **11** had IC_{50} values of 169.3 and 21.9 μM respectively. Isomers **12** and **17** had IC_{50} values of >146.4 and 32.6 μM, respectively.

Trimethoxy hybrid compounds **13** and **18** both showed biological activity at approximately 20 μM. Machilin G isoxazole analogues **14** and **15** exhibited IC_{50} values of 63.1 and 2.0 μM, respectively. Also, positional isomers **16** and **19** showed IC_{50} measurements of 3.3 and 9.5 μM respectively.

Among the compounds obtained, those with antileishmanial activity below 20 μM were tested against the promastigotes of *L. braziliensis*, related to cases of mucocutaneous leishmaniasis, to determine whether the isoxazole compounds could demonstrate biological activity against other *Leishmania* species. Positional isomers **15**, **16** and **19** were the most active compounds against *L. braziliensis,* showing IC_{50} values of 1.2, 2.1 and 6.4 μM, respectively, and cytotoxicity >250 μM (Table 2).

Compounds **15**, **16** and **19** were more active than the reference drug pentamidine for the two species of *Leishmania* studied.

Grandisin showed an IC_{50} = 28 μM against the promastigote forms of *L. amazonensis* while its derivative, the isoxazole analogue **20**, showed higher activity (IC_{50} = 18.3 μM), indicating that the presence of this nucleus improved the antileishmanial activity (Table 2).

The selectivity index, a crucial parameter in all drug-design studies, was established as the ratio of IC_{50} for mammal cells (NIH/3T3) to the IC_{50} value of protozoans treated with synthetic compounds obtained in an anti-trypanosomatid assay. Higher selectivity indexes correspond to increased specificity and selectivity to parasites.[34-35]

Synthetic compounds **15** and **16** were non-cytotoxic against mammal cells, with optimal selectivity index (SI>75.8 and SI>125.0, respectively), when compared to first-line drugs such as pentamidine (SI = 7.8), which has a higher cytotoxicity on murine fibroblast cells (NIH/ 3T3) (Table 2). Concerning the structure-activity relationship (SAR), isoxazole analogues structured with methylenedioxy exhibited impressive potency, highlighted by the machilin G isoxazole derivative **15** and positional isomers **16** and **19**, hybrids between grandisin **2** and machilin G **3**. The bioisosteric replacement of the tetrahydrofuran nucleus by the isoxazole ring in **1-3**, increased potency and selectivity of the synthesized compounds over *Leishmania* species. These results showed similar behaviour, to the replacement of this same core with the 1,2,3-triazole ring.[2]

3. Conclusions

In conclusion, 16 isoxazole analogues derived from neolignans **1–3** were synthesized. The most-active compounds **15**, **16** and **19**, which contain the methylenedioxy group, appear to be essential for anti-leishmanial activity. The anti-leishmanial activity of synthesized 3,5-diaryl-isoxazoles, described herein, was similar to 1,4-diaryl-1,2,3-triazoles,[2] indicating that these rings are equivalents and interchangeable.

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Conflicts of interest

There are no conflicts to declare.

FIGURE LEGENDS

Figure 1. Tetrahydrofuran neolignans veraguensin **1**, grandsin **2** and machilin G **3.**

Figure 2. Design of 1,4-diaryl-1,2,3-triazole[2,8] and 3,5-diaryl isoxazole analogues based on tetrahydrofuran neolignans **1-3**

Scheme 1. Synthesis of aryl acetylenes **26a-d**. Reagents and reaction conditions: (a) NBS, *p*-TsOH, CH_2Cl_2, SiO_2, **22a =** 81%, **22b** = 87%, **22c** = 79%; (b) 2-methyl-3-butyn-2-ol, $PdCl_2(PPh_3)_2$/CuI, Et_3N, reflux, 24h, **23a** = 85%, **23b** =75%, **23c = 79%**; (c) KOH, toluene, reflux, 24h, **26a** = 65%, **26b** = 85% , **26c** = 81%; (d) CBr_4, PPh_3/ CH_2Cl_2, 0°C, 5h., **25** = 74%; (e) THF, *n*-BuLi, -25 °C to room temperature, 1h, **26d** = 83%.

Scheme 2. Synthesis of Chloro-oximes **29a-c**. Reagents and reaction conditions: (a) $NH_2OH.HCl$ / KOH, H_2O / EtOH 1:1, **28a** = 85%, **28b** = 95%, **28c** = 95%; (b) NCS, DMF, 0 °C, 2 h, **29a** = 72%, **29b** = 75%, **29c** = 83%.

Table 1. Synthesis of 3,5-diaryl-isoxazole **5-16.**

Scheme 3. Synthesis of oxime **30**.

Scheme 4. Synthesis of isoxazoles **17-20**. Reagents and reaction conditions: (a) NCS, $CHCl_3$, pyridine, Et_3N, rt, 48 h; yields, **17** = 49%; **18** = 51%; **19** = 45%; **20** = 43%.

Table 2. *In vitro* anti-trypanosomatid activity of 3,5-diaryl-isoxazole analogues **5–20**.

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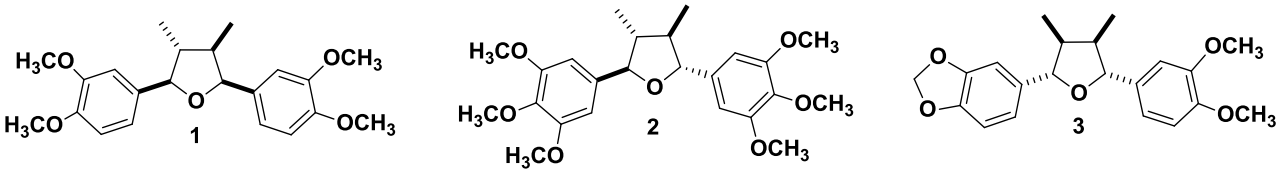


Figure 1. Tetrahydrofuran neolignans veraguensin **1**, grandsin **2** and machilin G **3.**

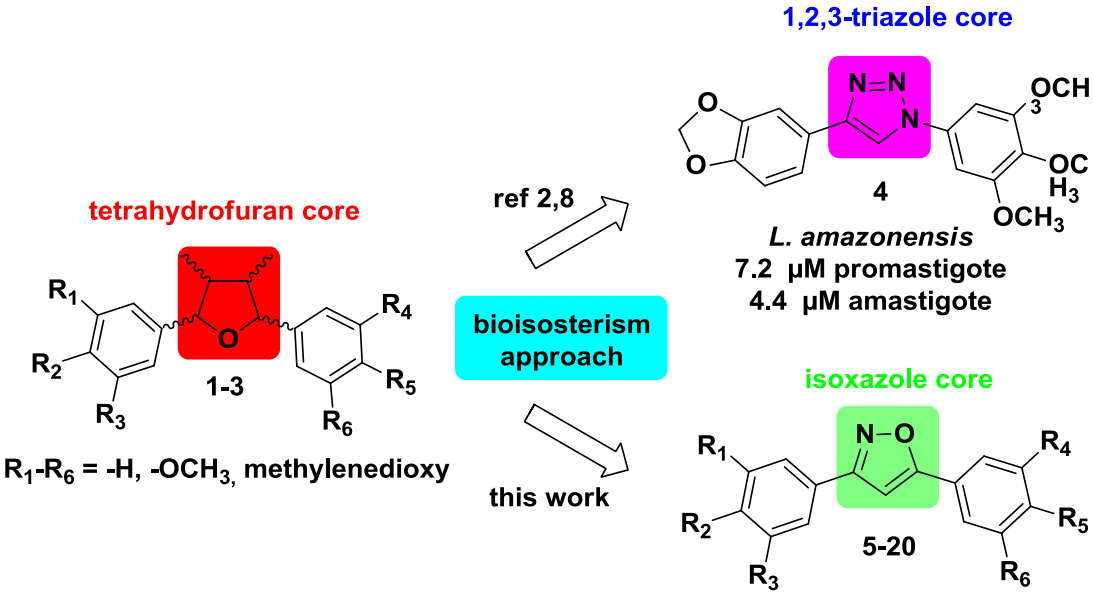
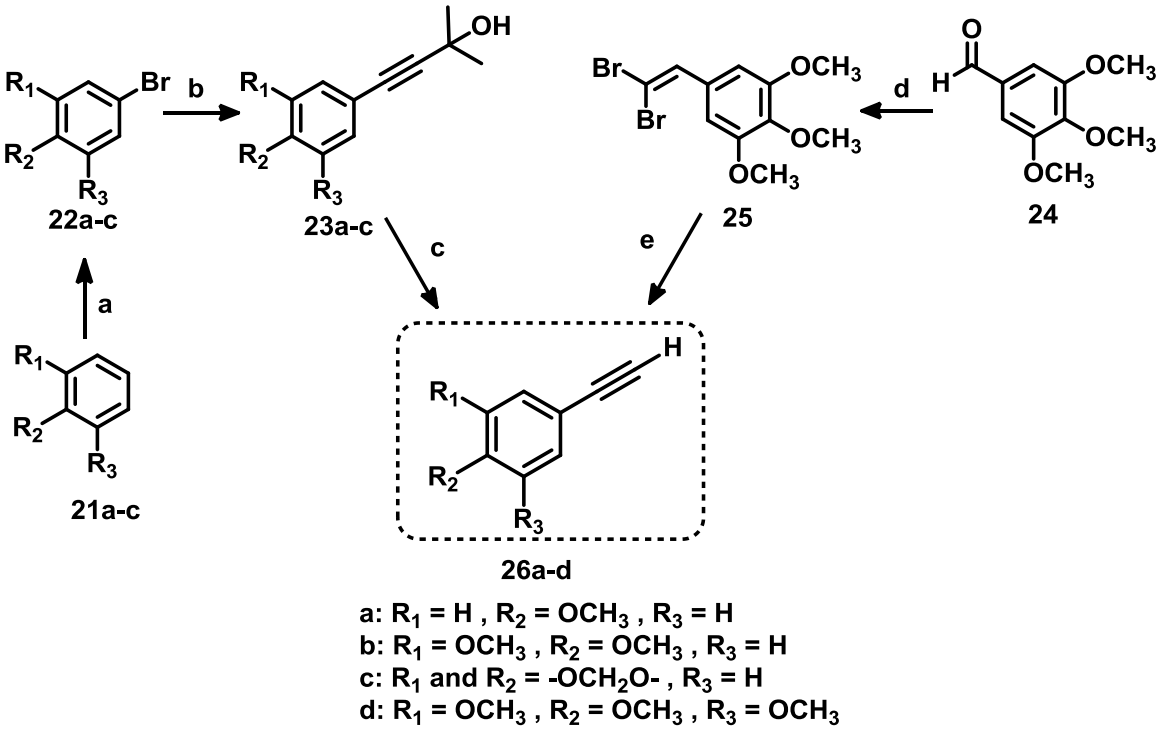
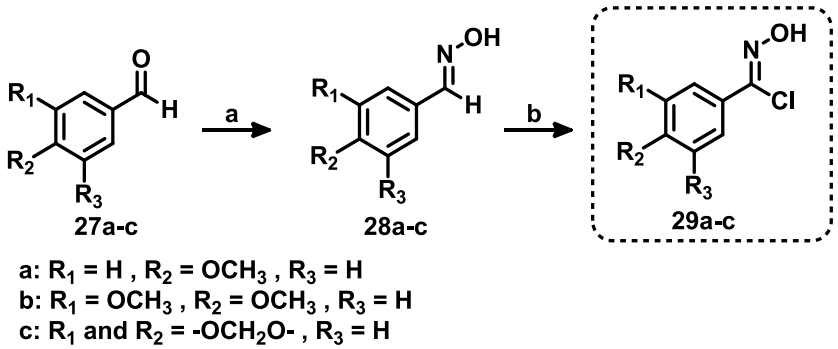


Figure 2. Design of 1,4-diaryl-1,2,3-triazole[2,8] and 3,5-diaryl isoxazole analogues based on tetrahydrofuran neolignans **1-3**

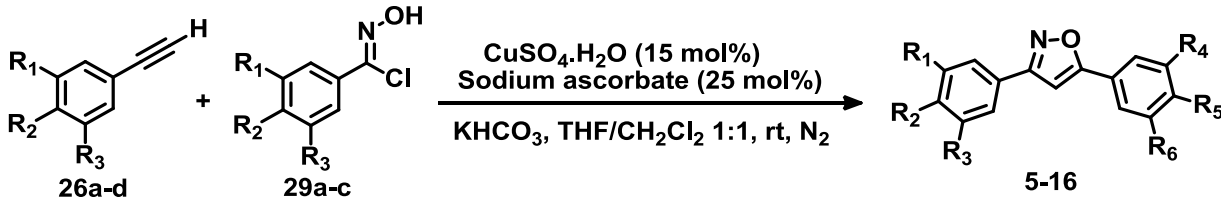


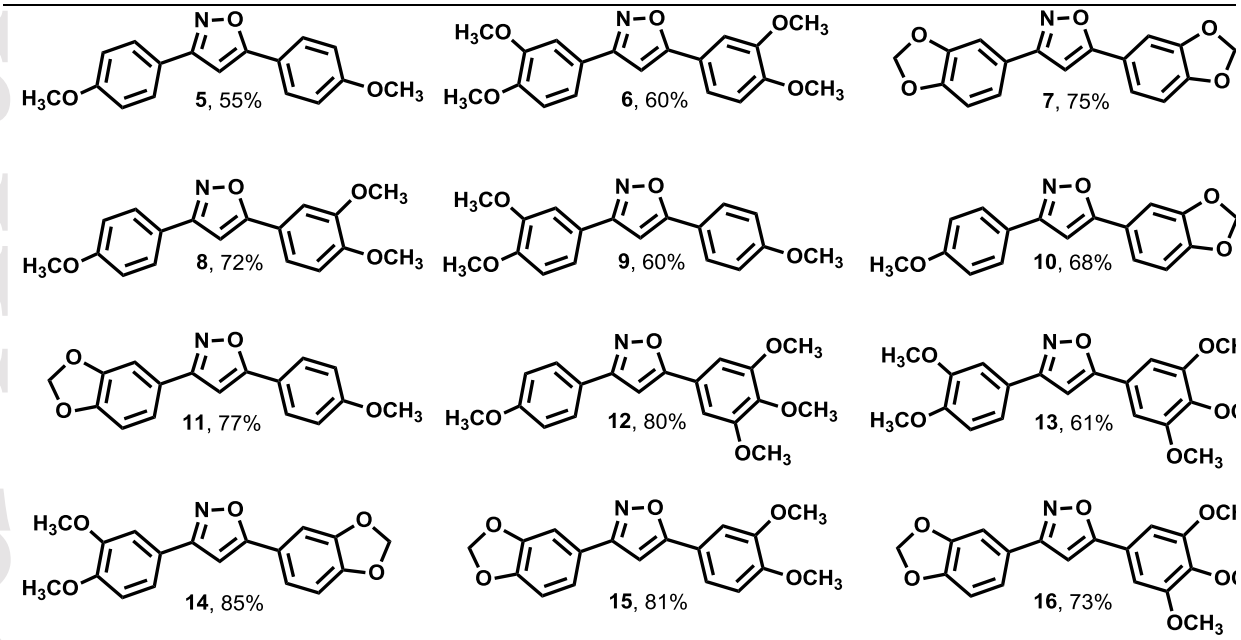
Scheme 1. Synthesis of aryl acetylenes **26a-d**. Reagents and reaction conditions: (a) NBS, *p*-TsOH, CH_2Cl_2, SiO_2, **22a =** 81%, **22b** = 87%, **22c** = 79%; (b) 2-methyl-3-butyn-2-ol, $PdCl_2(PPh_3)_2$/CuI, Et_3N, reflux, 24h, **23a** = 85%, **23b** =75%, **23c = 79%**; (c) KOH, toluene, reflux, 24h, **26a** = 65%, **26b** = 85% , **26c** = 81%; (d) CBr_4, PPh_3/ CH_2Cl_2, 0°C, 5h., **25** = 74%; (e) THF, *n*-BuLi, -25 °C to room temperature, 1h, **26d** = 83%.



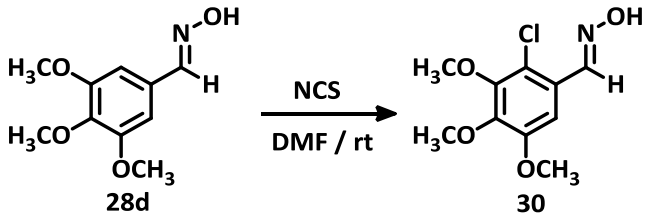
Scheme 2. Synthesis of Chloro-oximes **29a-c**. Reagents and reaction conditions: (a) $NH_2OH.HCl$ / KOH, H_2O / EtOH 1:1, **28a** = 85%, **28b** = 95%, **28c** = 95%; (b) NCS, DMF, 0 °C, 2 h, **29a** = 72%, **29b** = 75%, **29c** = 83%.

Table 1. Synthesis of 3,5-diaryl-isoxazole **5-16.**

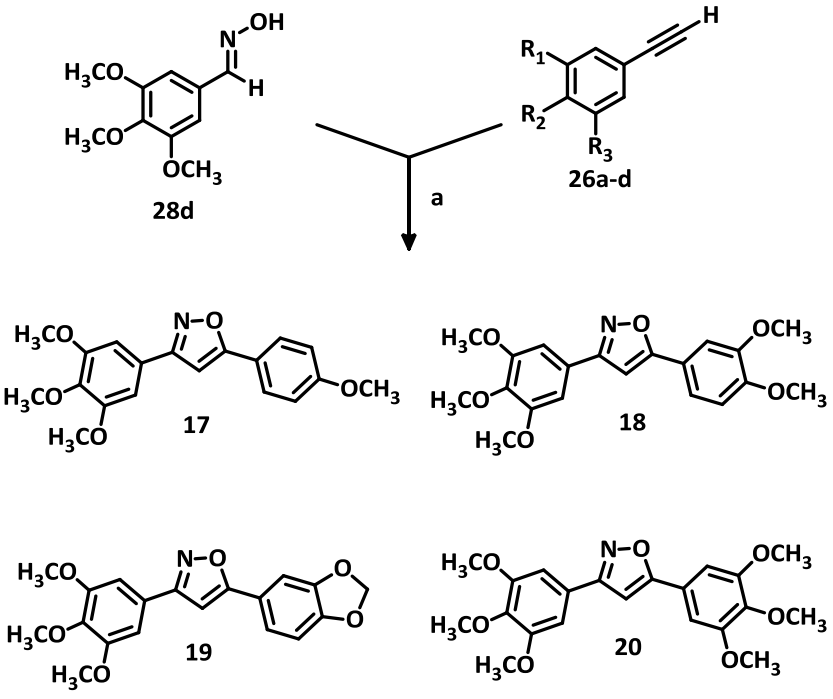




[a]Reactions conditions: **26a-d** (2.0 mmol), **29a-c** (2.0 mmol), $CuSO_4.5H_2O$ (0,30 mmol), sodium ascorbate (0,50 mmol), $KHCO_3$ (20 mmol), THF (3 mL), CH_2Cl_2 (3 mL), room temperature, 24h.



Scheme 3. Synthesis of oxime **30**.



Scheme 4. Synthesis of isoxazoles **17-20**. Reagents and reaction conditions: (a) NCS, $CHCl_3$, pyridine, Et_3N, rt, 48 h; yields, **17** = 49%; **18** = 51%; **19** = 45%; **20** = 43%.

Table 2. *In vitro* anti-trypanosomatid activity of 3,5-diaryl-isoxazole analogues **5–20**.

Compd	NIH/3T3 IC_{50}[b] / (μM) ± SD	*T. cruzi* IC_{50}[b] / (μM) ± SD	SI[c]	*L. amazonensis* IC_{50}[b] / (μM) ± SD	SI[c]	*L. braziliensis* IC_{50}[b] / (μM) ± SD	SI[c]
5	>250	>200	NC[d]	177 ± 2.2	>1.4	NC[d]	NC[d]
6	>250	>200	NC[d]	34.0 ± 1.5	>7.4	NC[d]	NC[d]
7	>250	>200	NC[d]	13.9 ± 1.1	>18	NC[d]	NC[d]
8	>250	>200	NC[d]	200 ± 2.3	NC	NC[d]	NC[d]
9	>250	>200	NC[d]	160 ± 2.2	>4.2	NC[d]	NC[d]
10	>250	NC[d]	NC[d]	169 ± 2.2	>9.4	NC[d]	NC[d]
11	>250	>200	NC[d]	21.9 ± 1.3	>11.4	NC[d]	NC[d]
12	>250	>200	NC[d]	146 ± 2.1	>11.2	NC[d]	NC[d]
13	>250	NC[d]	NC[d]	20.2 ± 1.3	>12.4	NC[d]	NC[d]
14	>250	183.6 ± 2.2	NC[d]	63.1 ± 1.8	>4.0	NC[d]	NC[d]
15	>250	NC[d]	NC[d]	2.0 ± 0.3	>125	1.2 ± 0.0	>208
16	>250	>200	NC[d]	3.3 ± 0.5	>75.8	2.1 ± 0.3	>119
17	>250	>200	NC[d]	32.6 ± 1.5	>7.7	NC[d]	NC[d]
18	>250	>200	NC[d]	19.4 ± 1.2	>12.9	NC[d]	NC[d]
19	>250	153.2 ± 2.1	NC[d]	9.5 ± 0.9	>20	6.4 ± 0.8	>39
20	>250	>200	NC[d]	18.3 ± 1.2	>14.2	25.2± 1.4	>9.9
PE[e]	78.7 ± 1.9	NC[d]	NC[d]	8.9 ± 0.9	8.8	10.1 ± 1.0	7.8
GRAN[f]	>250	NC[d]	NC[d]	28.0 ± 1.4	8.9	NC[d]	NC[d]
BZN[g]	96.1 ± 1.9	7.3 ± 0.8	13.2	NC[d]	NC[d]	NC[d]	NC[d]
DOX[h]	2.6± 0.4	NC[d]	NC[d]	NC[d]	NC[d]	NC[d]	NC[d]

[a] IC_{50}: half maximal inhibitory concentration on fibroblast cells; [b] IC_{50}: half maximal inhibitory concentration on *T. Cruzi, L. amazonensis* and *L. braziliensis;* [c] SI: selectivity index: IC_{50} on mammal cells per IC_{50} on trypanosomatids; [d] NC: values not calculated; [e] PE = pentamidine, positive control for *L. Amazonensis* and *L. braziliensis;* [f] GRAN = grandisin, positive control of natural product for *L. amazonensis* ; [g] benznidazole, positive control for *T. Cruzi;* [h] doxorubicin positive control for fibroblast cells.