Orbital: The Electronic Journal of Chemistry
journal homepage: www.orbital.ufms.br
e-ISSN 1984-6428

| Vol 12 | | No. 4 | | October-December 2020 |

FULL PAPER

Sustainable Synthesis of Novel Arylamide L-Cysteine Methyl Esters Peptidomimetic Derivatives: Inhibitors of Serine and Cysteine-like Proteases

Aline Silveira Serralbo[a], Daniel de Carvalho Santos[a], Marghuel Aparecida Vieira Silveira[b], Débora Noma Okamoto[c], Maria Aparecida Juliano[b], Suzan Pantaroto de Vasconcellos[c], Adriana Karla Cardoso Amorim Reis[a]*

[a]Universidade Federal de São Paulo, Departamento de Química, Instituto de Ciências Ambientais, Químicas e Farmacêuticas. Rua Prof. Artur Riedel, 275 - Jd. Eldorado - 09972-270 – Diadema, SP – Brasil.
[b]Universidade Federal de São Paulo, Departamento de Biofísica, Instituto de Farmacologia de Biologia Molecular. Rua Três de Maio, 100, Vila Clementino, 04044020 - São Paulo, SP – Brasil.
[c]Universidade Federal de São Paulo, Departamento de Ciências Farmacêuticas, Instituto de Ciências Ambientais, Químicas e Farmacêuticas. Rua São Nicolau, 210, Centro - 09913030 - Diadema, SP – Brasil.

Article history: Received: 04 December 2020; accepted: 31 December 2020. Available online: 31 December 2020.
DOI: http://dx.doi.org/10.17807/orbital.v12i4.1575

Abstract:

The development of new protease inhibitors is always expanding for active therapies against diseases caused by pathogenic microorganisms that rely on proteases for replication and vital functions. This work aimed to synthesize new peptidomimetics arylamides, endeavoring to provide these compounds with the capacity to inhibit mainly cysteine and serine-like proteases. The effectiveness of COMU as coupling reagent, under classical and sustainable approaches, were evaluated. The results confirmed that the use of dichloromethane and the classical methodology is efficient for new amide bond formation in terms of yield. Although, the use of dimethyl carbonate and the microwave-assisted methodology proved competitive performance and can be used as an alternative route due to its environmentally friendly approach (green solvent and energy efficiency). In vitro screening assays attested that the proposed compounds have inhibitory activity for papain (IC_{50} between 5.56±0.21 μM and 18.50±0.69 μM) and β-trypsin (IC_{50} between 250±12 μM and 1410±30 μM). The compounds **1a** and **1e** were noteworthy with IC_{50} values of 5.56±0.21 μM and 7.06±0.33 μM for papain and 540±10 μM and 250±12 μM for β-trypsin, respectively.

Keywords: arylamides; peptidomimetics; coupling reactions; green solvents; protease inhibitors

1. Introduction

Given the crescent necessity for the development of new drugs using efficient and green synthetic routes, the demand for alternative methods has been of paramount importance for the obtention of bioactive compounds. Synthetic routes aiming to preserve natural resources and minimize by-products while sustaining the same quality of medicines has been one of the main focuses of synthetic organic chemistry [1].

The use of coupling reagents for amidation is a strategy that has been adopted to substitute the use of acyl chlorides since they are hazardous and can cause several problems for the environment and human health [2, 3]. Due to several years of research and studies, these methodologies proved to be quite efficient in obtaining new amide bonds and also, can be used as a substituent for sustainable approaches

A multitude of coupling reagents have been developed, often promising to improve performance and comparing the efficacy of

*Corresponding author. E-mail: adrianaamorim@unifesp.br

coupling reagents, which is not a trivial task due to many parameters, influencing amide bond formation [4]. Nevertheless, over the past two decades, benzotriazole-based coupling reagents like HBTU [5] and HCTU [6, 7] have emerged as very cost-efficient coupling reagents. These coupling reagents have found applications in automated synthesis [8-12], also at industrial peptide production processes [4, 13]. However, the recent reclassification of 1-hydroxybenzotriazole as an explosive compound, has led to a search for alternative coupling reagents that do not contain the potentially explosive benzotriazole moiety [14].

COMU [(1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino carbenium hexafluorophosphate] is a third generation of uronium-type coupling reagent based on ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) as well as amorpholino carbon skeleton (Figure 1) [15].

COMU showed greater coupling efficiency, reduced epimerization and a less hazardous safety profile [15]. It has been introduced as a nonexplosive alternative to the classical benzotriazole coupling reagents. The coupling efficiency of COMU is superior to both HBTU and HCTU and, often, also to the relatively expensive gold standard HATU [16]. Varying solution stabilities of the novel coupling reagent COMU have been reported [17].

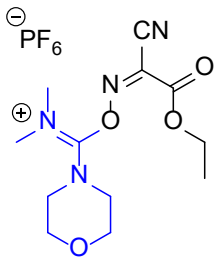


Figure 1. COMU coupling reagent structure.

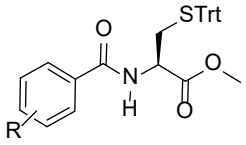
The most common solvents employed in amide coupling reactions must be dipolar aprotic solvents, such as NMP (*N*-methyl-2-pyrrolidinone), DMF (*N,N'*-dimethylformamide) and DMAc (*N,N'*-dimethylacetamide), which have environmental issues [18]. Chlorinated solvents such as CH_2Cl_2 (dichloromethane), can be common alternatives, but still offers safety issues, alongside increased disposal costs [18-20].

Organic carbonates, such as dimethyl carbonate (DMC), diethyl carbonate (DEC) or ethylene carbonate (EC), offer various advantages as green solvents for organic transformations [19]. They are available in large amounts and at low prices; having low ecotoxicity and are highly biodegradability [20].

Protease inhibitors (PIs) can be broadly classified according to their structure into two major groups: low molecular weight inhibitors and peptide inhibitors with one or more polypeptide chains [21]. These inhibitors can further be divided according to the type of protease they inhibit (i.e. serine, cysteine, aspartyl or metallo-protease inhibitors) [21-23].

Peptidomimetics are designed to circumvent some of the problems associated with natural peptides, such as poor stability against proteolysis (duration of activity) and poor bioavailability. In addition, selectivity and potency can also be substantially improved [24, 25]. Several peptidomimetic compounds have been already approved as important drugs or are in late-stage clinical trials, with applications in the treatment for different pathologies, spanning from infectious diseases to cancer and rare diseases [24-27].

The pathophysiological importance of proteases as molecular targets has boosted the research and development of new protease inhibitors over the last decades [22]. Hence, given the peptide nature of protease inhibitors, finding new synthetic methods of amide synthesis has become a central issue for the pharmaceutical industries, in order to ease the handling of products and reactants, develop simpler reaction conditions and achieve automated experimental procedures [28, 29].



1a R = H
1b R = *m*-MeO
1c R = *p*-MeO
1d R = *o*-MeO
1e R = *m*-NO_2
1f R = *p*-NO_2
1g R = *o*-NO_2

1a-g Trt = Trityl

Figure 2. Proposed arylamides (**1a-g**).

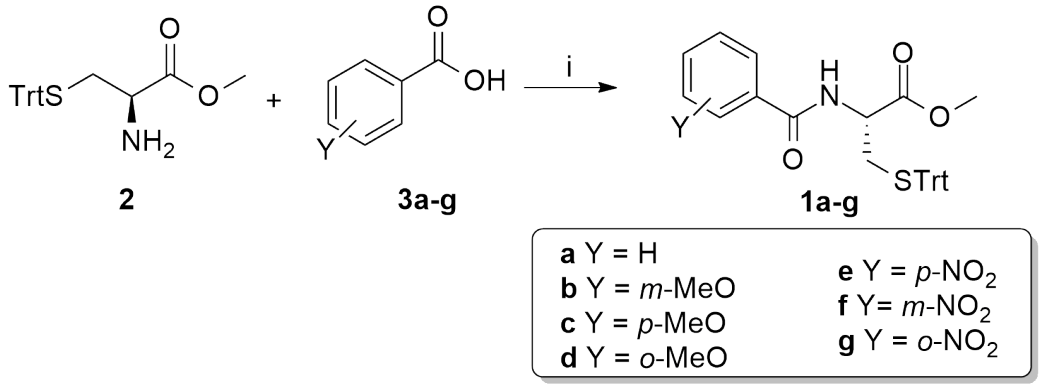
In line with our interest in the development of sustainable methods for the synthesis of

biologically active organic compounds, here we present the study on the synthesis of arylamides (**1a-g**, Figure 2) using COMU coupling reagent and dichloromethane dimethyl carbonate as a green solvent. Additionally, the inhibitory activity of the title compounds was evaluated for cysteine and serine-like proteases (i.e. papain and β-trypsin, respectively).

2. Results and Discussion

2.1 Synthesis of L-cysteine-esters arylamides derivatives

The preparation of the arylamides (**1a-g**) was carried out by reacting the *S*-trityl-*L*-cysteine methyl ester with benzoic acids with different substituent groups (**3a-g**) (Scheme 1).



Scheme 1. Reaction between *S*-trityl-*L*-cysteine methyl ester with benzoic acids with different substituents. ***Reagents & Conditions:*** (i) COMU, CH_2Cl_2 or DMC, conventional heating or MW.

Two synthetic approaches were studied, using conventional and sustainable methodologies, in order to establish the scope and limitations of the methods. COMU was used as coupling reagent, using classical conditions described elsewhere [30, 31]. Thus, the use of COMU as coupling reagent led to the L-cysteine-esters arylamides derivatives (**1a-g**) in yields varying from 48% to 63%, confirming the successfulness of the reaction (Table 1). Nevertheless, it is known that COMU shows coupling efficiency and a safer profile than the first- and second-generation coupling reagents [32].

Next, we investigate the use of microwave as alternative source of heating in the reaction of *S*-trityl-L-cysteine methyl ester (**2**) and benzoic acid (**3a**) under different conditions (Scheme 2, Table 2). The best microwave-assisted conditions for this model reaction were find when COMU was used as coupling reagent and dichloromethane as solvent. Firstly, the reaction was carried out at 60 °C, varying the time (15-45 min), and the product **1a** was obtained in very low yields (entries 1-4). When the temperature was increased to 80 °C, during 30 minutes, it was noticed a significant increase in the reaction yield (entry 5). Finally, the same reaction was performed at higher temperatures (100 °C and 120 °C) during 30 minutes, with no significant improvement in the yield for **1a** (entries 6 and 7).

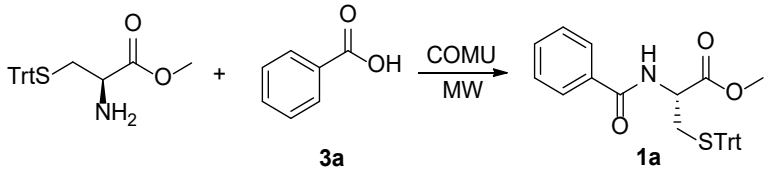
Table 1. Yields obtained with the coupling reaction using COMU in CH_2Cl_2.

entry [a]	Compound	Yield (%)
1	1a	48
2	1b	59
3	1c	49
4	1d	51
5	1e	63
6	1f	50
7	1g	59

Reagents and conditions: [a] 0.6 mmol of *S*-trityl-*L*-cysteine methyl ester, 0.6 mmol of **3a-g**, 0.66 mmol of COMU, 15 mL of CH_2Cl_2, 40ºC, 6h. Isolated yields.

The use of dimethyl carbonate (DMC) as an alternative solvent to dichloromethane is already well known [33, 34]. Once the optimum condition was established for the preparation of **1a** (Table 2, entry 5), we next proceeded with the evaluation of the reactions under microwave heating, using two different solvents (dichloromethane and dimethyl carbonate). Microwave-assisted coupling reactions for the synthesis of amides have experienced an increased interest over the past years [35, 36].

Table 2. Control experiments for **1a** model reactions using microwave methodology.



3a **1a**

entry[a]	Temperature (°C)	Time (min)	Yield (%)*
1	60	15	13
2	60	20	24
3	60	30	35
4	60	45	23
5	80	30	57
6	100	30	55
7	120	30	48

Reagents and conditions: [a] 0.08 mmol of *S*-trityl-*L*-cysteine methyl ester, 0.08 mmol of **3a**, 0.08 mmol of DCC, 5 mL of CH_2Cl_2; *all reactions were performed in a sealed tube under microwave irradiation using MONOWAVE 300® (Anton Paar) reactor. Isolated yields.

In this scenario, we have compared the yields obtained for arylamides **1a-g** in the reaction of *S*-trityl-L-cysteine methyl ester with benzoic acids (**3a-g**) with COMU in microwave-assisted reaction using CH_2Cl_2 (Table 3, entries 1-7) and DMC (entries 8-14) as solvents. The use of microwave-assisted methodology, by itself, raised the conversion of product yields when compared to entries in Table 1. This was more evident employing COMU, where the use of CH_2Cl_2 in microwave media maintained or increased the yields (Table 3) due to the enhanced reactivity of this kind of coupling reagent in these conditions.

Overall, the yields obtained with the use of dimethyl carbonate were slightly lower when compared to those obtained when dichloromethane as solvent, regardless the coupling reagent employed. However, despite higher conversion rates observed for the reactions carried out in dichloromethane, isolation of the compounds was hampered, since the byproducts are quite soluble in this solvent.

Table 3. Yields obtained with the coupling reactions via COMU by microwave (closed flask) in dichloromethane or dimethyl carbonate medium.

entry [a]	Compound	Solvent	Yield (%) *
1	1a	CH_2Cl_2	57
2	1b	CH_2Cl_2	66
3	1c	CH_2Cl_2	70
4	1d	CH_2Cl_2	44
5	1e	CH_2Cl_2	66
6	1f	CH_2Cl_2	52
7	1g	CH_2Cl_2	62
8	1a	DMC	47
9	1b	DMC	47
10	1c	DMC	32
11	1d	DMC	39
12	1e	DMC	47
13	1f	DMC	47
14	1g	DMC	47

Reagents and conditions: [a] 0.08 mmol of *S*-trityl-*L*-cysteine methyl ester, 0.08 mmol of **3a-g**, 0.08 mmol of COMU, 5 mL of CH_2Cl_2 or DMC, 80°C, 30 min; *all reactions were performed in a sealed tube under microwave irradiation using MONOWAVE 300® (Anton Paar) reactor. Isolated yields

2.2 Protease Inhibition Assays

The inhibitory activity of all synthesized aryl amides was evaluated in vitro, through the quantification of the half-maximal inhibitory concentration (IC_{50}), using the papain and β-trypsin (cysteine and serine proteases, respectively) as models. To guarantee the reliability of the results, the purity of the compounds used was maintained above 95% (HPLC analysis).

The peptidases were tested at their pH of maximal activity and previously incubated with all the inhibitors. The specific substrate for each of the proteolytic activities was added to 6 μM (final concentration) to guarantee the best speed during the experiment.

Firstly, an initial analysis was performed using a range of concentrations based on well-known

commercial inhibitors (E-64 and PMSF) [37]. For papain, the initial screening was done using concentrations between 1 to 30 μM, in which it was noticed that our compounds did not show any considerable inhibitory activity until 25 μM. Moreover, for β-trypsin, the initial values used were between 100 and 600 μM, and, likewise, the compounds did not show inhibitory activity up to 500 μM. Therefore, the concentrations were increased in order to obtain the IC_{50} values for these types of proteases.

The results of the screening with higher concentrations are shown in Tables 4 and 5. The compounds **1a** (R=-H) and **1e** (R=*p*-NO_2) showed the most relevant inhibitory activity in both cysteine-like and serine-like induced assays, being those values smaller than the standards (E-64 and PMSF). Satisfactory results for β-trypsin were also obtained for compounds **1c** (R=*p*-MeO) and **1f** (R=*m*-NO_2), which had an IC_{50} of 660±21 μM and 620±14 μM, respectively.

Table 4. IC_{50} (μM) of *N*-substituted-methyl-2-benzamido-3-(tritylthio)-propanoate derivatives (25–70 μM) on papain (cysteine-like protease) inhibition.

Compounds	R	IC_{50} (μM)
1a	-H	5.56±0.21
1b	*m*-MeO	11.33±0.52
1c	*p*-MeO	18.50±0.69
1d	*o*-MeO	16.78±0.55
1e	*p*-NO_2	7.06±0.33
1f	*m*-NO_2	17.01±0.60
1g	*o*-NO_2	17.33±0.59
E-64	-	6.64±0.25

Table 5. IC_{50} (μM) OF *N*-substituted-methyl-2-benzamido-3-(tritylthio)-propanoate derivatives (500–5000 μm) on β-trypsin (serine-like protease) inhibition.

Compounds	R	IC_{50} (μM)
1a	-H	540±10
1b	*m*-MeO	950±19
1c	*p*-MeO	660±21
1d	*o*-MeO	1410±30
1e	*p*-NO_2	250±12
1f	*m*-NO_2	620±14
1g	*o*-NO_2	830±18
PMSF	-	670±15

The values of IC_{50} showed that the compound **1a** was active in relatively low concentration for the inhibition of papain. The insertion of an electron-withdrawing group (R= NO_2) at the *para* position of *N*-benzoyl moiety (**1e**) had an important contribution to potentializing the products inhibitory property, probably due to the decrease in the electron density in the ring, which might lead to greater interaction via π-stacking with the catalytic site amino acids. Complementarily, the introduction of an electron donor group (R= MeO) surrounding the pharmacophoric aromatic ring was not able to improve the protease inhibition profile.

For β-trypsin, it is important to notice that the lack of substituents in the ring (**1a**) positively influences inhibition, being with values close to the PMSF. Just as before, the presence of the nitro group in the para position of the ring (**1e**) proved to be beneficial when compared to the rest of the series. These data reveal that the presence of an electron withdrawing group is likely to be important for intermolecular interactions with serine-like proteases. Compounds **1d** and **1g** did poorly in inhibiting β-trypsin, indicating that the presence of substituents in ring ortho position can cause impediment in the side-chain attachment with β-trypsin. However, when the substituent is in *meta* (**1c** and **1f**) the inhibition rate seems to be positively affected, remaining close to the standards.

3. Material and Methods

3.1 General

Solvents used to carry out this work were obtained commercially from Synth. Reagents were purchased from the following suppliers: Acros®, Merck®, Fluka®, Sigma Aldrich®. The anhydrous solvents were treated according to the literature: (tetrahydrofuran: pre-treatment with potassium hydroxide (KOH), further treated with sodium metal and as indicator was used benzophenone); (dichloromethane (CH_2Cl_2): Distilled and dried with calcium hydride (CaH)); (triethylamine: Distilled and then dried with calcium hydride). According to the need for the experiments, the reagents were treated as described in the literature together with the anhydrous operations.

General purification of the compounds was performed by column chromatography using flash silica gel (200-400 mesh) Aldrich brand. Reaction monitoring was performed by thin layer chromatography (CCD) on silica gel chromatograms Merck 60 F254 supported on aluminium plates (with fluorescein). The visualization of the compounds was carried out with phosphomolybdic acid, UV-light and *p*-anisaldehyde.

Microwave experiments were carried out using MONOWAVE 300 microwave reactor (Anton Paar®), operating at 2.455 GHz frequency with continuous irradiation power from 0 to 300 W; G4 and G10 borosilicate glass vials (manufacturer design), sealed with Teflon septum, were used for reactions in a 0.5 mmol and >1.0 mmol scale, respectively. All described reaction times reflect the irradiation time at the set reaction temperature.

During the laboratory operations the Büchi® R-210 rotoevaporators were coupled with a Büchi® V-700 pump with V-850 vacuum controller and Heidolph® Hei-VAP Precision, coupled with a Valve Control vacuum pump for the removal of solvents volatiles.

For the full extraction of traces of solvents, the high vacuum pump Edwards® V-820 was used. For the weighing of the reagents, the analytical balance Sartorius® model CPA22D was used.

The identifications of the compounds were done at the Analytical Centre of the Institute of Chemistry of the Universidade de São Paulo-IQ-USP and at the Analytical Centre of the Universidade Federal de São Paulo - Campus Diadema. ^{1}H and ^{13}C NMR spectra were obtained on Brucker™ spectrometers; model Ultra-shield 300, Advance III 300 console, operating for ^{1}H at 300 MHz and ^{13}C at 75 MHz using deuterated chloroform ($CDCl_3$) as solvent, and as reference standard tetramethylsilane (TMS).

High resolution mass spectrometry (HRMS) analyses were carried out using a Bruker MicroTOF 61 spectrometer (electrospray ionization, ESI (+)). Melting points were determined on a Büchi Melting Point M-565 apparatus.

3.2 Synthesis of compounds

*3.2.1 Reaction using conventional conditions for preparing (R)-methyl 2-benzamido-3-(tritylthio) propanoates (**1a-g**).*

Into a round bottom flask under magnetic stirring, the correspondent benzoic acid (73.3 mg, 0.6mmol), *S*-trityl-*L*-cysteine methyl ester (226.5 mg, 0.6mmol), COMU (282.6 mg, 0.66mmol) and 15 mL dichloromethane were added and refluxed for 6 hours at a temperature of 40°C. After the reaction time, the solution was allowed to cool and then it was filtered. Then, the solvent was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 9:1 to 1:1) resulting in compound **1a**, a white solid. The other substituted compounds were performed with the same experimental procedure, the products range from yellow to orange oils depending on the benzoic acid used.

*3.2.2 Reaction using microwave-assisted conditions for preparing (R)-methyl 2-benzamido-3-(tritylthio) propanoate (**1a-g**).*

Into a microwavable flask under magnetic stirring, the correspondent benzoic acid (9.77 mg, 0.08 mmol), *S*-trityl-L-cysteine methyl ester (30.2 mg, 0.08mmol) and COMU (34.2 mg, 0.08mmol) were added, followed by the addition of 5 mL of dichloromethane or dimethyl carbonate. The mixture was allowed to reflux for 30 minutes at 80°C. After the reaction time, the solution was allowed to cool, then it was filtered and the solvent was removed under pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 9:1 to 1:1) resulting in compound **1a**, a white solid. The other compounds were done using the same experimental procedure, the products ranged from yellow to orange oils depending on the benzoic acid used.

*(R)-methyl 2-benzamido-3-(tritylthio)propanoate (**1a**):* CAS number: 309264-57-1; white solid; m.p. 130-133°C (lit. 132-134 °C); ^{1}H NMR (300 MHz, $CDCl_3$): δ= 2.83-2.71 (m, 2H),3.74 (s, 3H), 4.84 (sext, *J*=18 Hz, 1H); 6.72 (d, *J*=6 Hz, 1H), 7.55-7.15 (m, 18H), 7.78 (d, *J*=9 Hz, 2H);^{13}C NMR 75 MHz, ($CDCl_3$):δ= 34.07, 51.45, 52.78, 66.97, 126.92-133.69, 144.28, 166.84, 171.04; FTIR (cm^{-1}): 3422.25, 2913.93, 2846.10,

1744.22, 1646.27, 1599.72, 1545.22, 1488.69, 1444.88, 1385.56, 742.78, 696.23; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 482.1784; found: *m*/*z* 482.1783.

*(R)-methyl 2-(4-methoxybenzamido)-3-(tritylthio)propanoate (**1b**):* CAS number: 309264-62-8; pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): *δ*= 2.70-2.81 (m, 2H), 3.74 (s, 3H), 3.86 (s, 3H), 4.83 (sext, *J*= 15 Hz, 1H), 6.61 (d, *J*= 9 Hz, 1H), 6.92 (d, *J*= 9 Hz, 2H), 7.18-7.39 (m, 16H), 7.72 (d, *J*= 9 Hz, 1H) ;^{13}C NMR 75 MHz, ($CDCl_3$): *δ*= 34.17, 51.36, 52.73, 55.45, 66.91, 113.76, 125.97-129.49, 144.30, 162.50, 166.30, 171.18; FTIR (cm^{-1}): 3428.63, 2927.49, 2833.53, 1735.81, 1642.11, 1611.45, 1501.73, 1441.42, 1256.94, 1177.18, 1031.29, 849.72; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 512.1890; found: *m*/*z* 512.1889.

*(R)-methyl 2-(3-methoxybenzamido)-3-(tritylthio)propanoate (**1c**):* pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): *δ* =2.71-2.81 (m, 2H), 3.74 (s, 3H), 3.85 (s, 3H), 4.80 (sext, *J*= 18 Hz, 1H), 6.67 (d, *J*=9Hz, 1H), 7.05 (d, *J*=9 Hz, 1H), 7.16-7.40 (m, 18H);^{13}C NMR 75 MHz, ($CDCl_3$):*δ* = 34.03, 51.47, 52.77, 55.49, 66.96, 112.58, 118.10, 119.02, 126.92-129.39, 135.16, 144.27, 159.84, 166.70, 170.98; FTIR (cm^{-1}): 3428.65, 2927.49, 2859.66, 1744.65, 1641.13, 1573.30, 1484.01, 1320.55, 1278.03, 1241.33, 1038.64, 753.76, 706.68; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 512.1890; found: *m*/*z* 512.1892.

*(R)-methyl 2-(2-methoxybenzamido)-3-(tritylthio)propanoate (**1d**):* pale brown oil; ^{1}H NMR (300 MHz, $CDCl_3$): *δ* = 2.73-2.75 (m, 2H), 3.73 (s, 3H), 3.96 (s, 3H), 4.84 (q, *J*= 12 Hz, 1H), 6.98 (d, *J*= 9 Hz, 1H), 7.08 (t, *J*= 15 Hz, 1H), 7.16-7.50 (m, 16H), 8.15 (d, *J*= 9 Hz, 1H), 8.71 (d, *J*= 9 Hz, 1H);^{13}C NMR 75 MHz, ($CDCl_3$):*δ* = 34.17, 51.36, 52.73, 55.45, 66.91, 113.76, 125.97-129.49, 144.30, 162.50, 166.30, 171.18; FTIR (cm^{-1}): 3433.49, 2927.49, 2839.71, 1744.81, 1654.68, 1599.18, 1524.96, 1485.35, 1328.13, 1239.30, 1022.26, 752.20; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 512.1890; found: *m*/*z* 512.1893.

*(R)-methyl 2-(4-nitrobenzamido)-3-(tritylthio)propanoate (**1e**):* CAS number: 309264-61-7; orange oil; ^{1}H NMR (300 MHz, $CDCl_3$): *δ* =2.44-2.61 (m, 2H), 3.65 (s, 3H), 5.20 (q, *J*= 12 Hz, 1H), 7.18-7.48 (m, 19H), 8.04 (d, *J*= 12 Hz, 1H);^{13}C NMR 75 MHz, ($CDCl_3$):*δ* = 36.84, 52.21, 53.74, 66.89, 123.99, 126.81-130.08, 132.44, 139.40, 144.53, 150.34, 170.64, 174.10; ; FTIR (cm^{-1}): 3459.33, 2911.12, 2850.56, 1739.18, 1650.27, 1601.64, 1532.46, 1423.25, 1338.89, 1311.22, 1037.17, 837.28; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 527.1635; found: *m*/*z* 527.1635.

*(R)-methyl 2-(3-nitrobenzamido)-3-(tritylthio)propanoate (**1f**):* yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): *δ* =2.44-2.62 (m, 2H), 3.64 (s, 3H), 6.20 (q, *J*= 12 Hz, 1H), 7.17-7.44 (m, 16H), 8.09 (d, *J*= 12 Hz, 2H), 8.51 (d, *J*= 6 Hz, 1H), 8.76 (s, 1H);^{13}C NMR 75 MHz, ($CDCl_3$):*δ* = 36.94, 52.19, 53.81, 66.89, 124.10, 126.82-130.46, 132.78, 134.65, 144.57, 149.49, 169.73, 174.19; FTIR (cm^{-1}): 3428.68, 2921.11, 2846.10, 1742.17, 1639.00, 1595.64, 1514.04, 1380.95, 1350.99, 1321.42, 1084.65, 739.58, 696.22; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 527.1635; found: *m*/*z* 527.1636.

*(R)-methyl 2-(2-nitrobenzamido)-3-(tritylthio)propanoate (**1g**):* yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): *δ* =2.44-2.63 (m, 2H), 3.66 (s, 3H), 5.20 (quint, *J*= 18 Hz, 1H), 6.64 (d, *J*= 6 Hz, 1H), 7.19-7.44 (m, 16H), 7.61 (d, *J*= 9 Hz, 2H), 8.19 (t, *J*= 18 Hz, 1H);^{13}C NMR 75 MHz, ($CDCl_3$):*δ* = 36.89, 52.19, 53.76, 66.88, 122.95, 126.79-129.89, 131.32, 132.57, 135.26, 144.53, 146.37, 170.51, 174.16; ; FTIR (cm^{-1}): 3455.72, 2920.10, 2852.12, 1733.25, 1640.73, 1590.64, 1522.41, 1478.65, 1352.98, 1319.92, 1069.41, 769.54; HRMS (ESI) [*M*+H]+: calculated: *m*/*z* 527.1635; found: *m*/*z* 527.1633.

3.3 Enzymatic Inhibition Assays

3.3.1 Enzymes

Papain from Papaya was purchased from Sigma–Aldrich (C3142, batch-109H7485) and β-trypsin was purchased from Biobras Co. (Montes Claros, Minas Gerais, Brazil).

3.3.2 Peptide synthesis

All the FRET peptides were obtained by the solid-phase peptide synthesis strategy as previously described [38]. Stock solutions of peptides were prepared in DMSO, and the concentration measured spectrophotometrically using the molar extinction coefficient of EDDnp (ε_{365} = 17.300 $M^{-1}cm^{-1}$).

3.3.3 Hydrolysis of FRET peptides

he hydrolysis of FRET peptides was quantified using a *Molecular Devices Spectramax-190 Microplate Reader* spectrofluorometer by measuring the fluorescence at 420 nm following excitation at 320 nm. The inner-filter effect was corrected as previously described [39]. The concentration of DMSO in assay buffers was kept below 1%.

3.3.4 Inhibitory activity screening

The influence of all synthesized compounds on the catalytic activity of β-trypsin and papain was investigated. The tests were performed in 96-well plates in a plate spectrofluorometer.

The enzymatic assays were performed using the standard assay conditions described for each enzyme: enzymatic hydrolysis by papain in 50 mM Tris-HCl, pH 7.5 buffer. This enzyme was activated in the presence of 0.5 mM DTT incubated 10 minutes before adding the substrate. β-trypsin was incubated in 50 mM Tris-HCl, pH 7.5 buffer.

The enzyme concentrations to determine the hydrolysis rate were chosen at a level designed to linearly hydrolyse 1 mM of substrate added at the beginning of the reaction at 37ºC. For papain and β-trypsin, the sequence Abz-KLRSSKQ-EDDnp was used as substrate in a 6 μM buffer solution [37].

Commercial inhibitors E-64 and PMSF were used as a standard for comparison. Enzyme initial velocity of hydrolysis was measured after 10 minutes prior incubation of enzyme with the inhibitors at the following concentrations: from 25 μM to 70 μM for papain and 500 μM to 5000 μM for β-trypsin [37]. All experiments were performed in triplicate, in order to generate the standard error for each value. The IC_{50} and its respective standard deviations were calculated using the equation using Grafit® software (Erithacus Software, Horley, Surrey, UK).

4. Conclusions

One of the main advantages offered by microwave irradiation (which cannot be explained by thermal effects) derives from the dipolar polarization mechanism, which provides extra energy for the rotation of molecules with a dipolar moment, such as arylamides. We can state that the closed vessel microwave-assisted methodology has successfully responded to the coupling reactions to synthesize the arylamides of interest. Further, permeating environmental issues, such as the replacement of a conventional organic solvent such as CH_2Cl_2 by DMC, a solvent regarded as green and also showing that the reaction time decreased considerably compared with the reactions performed at conventional heating (energy efficiency).

Preliminary *in vitro* enzymatic assays showed that, in general, all compounds inhibited the analyzed proteases (papain and β-trypsin). Compounds **1a** and **1e** showed the best inhibition profile, with IC_{50} values equal or lower than the observed for the standards. Additionally, cell-based cytotoxicity assays will be performed in order to guarantee that these compounds are safe. Finally, the understanding of the molecular basis of the action of the studied inhibitors can be investigated by in silico techniques (i.e. molecular docking) to identify and understand the mode of interaction between the inhibitors and the proteases.

Supporting Information

Supplementary data associated with this article can be found in the online version: ^{1}H and ^{13}C NMR spectra and enzymatic assays.

Acknowledgments

The authors thanks to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - Grant Numbers: 2013/16644-4) for financial support. The authors also thank Coordenação Nacional de Aperfeiçoamento do Ensino Superior (CAPES 001- Brazil) for the research fellowships granted to A.S.S, D.N.O. and D.C.S.

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