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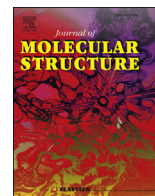


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Ultrasound-assisted green bromination of *N*-cinnamoyl amino acid amides – Structural characterization and antimicrobial evaluation



Boyka Stoykova^a, Maya Chochkova^{a,*}, Galya Ivanova^b, Nadezhda Markova^c,
Venelin Enchev^c, Iva Tsvetkova^d, Hristo Najdenski^d, Martin Štícha^e, Tsenka Milkova^a

^a Faculty of Mathematics and Natural Sciences, South-West University “Neofit Rilski”, 66, Ivan Mihailov Str., 2700, Blagoevgrad, Bulgaria

^b REQUIMTE-UCIBIO, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n, 4169-007, Porto, Portugal

^c Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 9, Sofia, 1113, Bulgaria

^d The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Building 26, Sofia, 1113, Bulgaria

^e Charles University, Faculty of Science, Section of Chemistry, Hlavova 2030/8, 12843, Prague 2, Czechia

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ABSTRACT

N-phenylpropenoyl amino acid amides have been brominated using two alternative sonochemically activated green chemistry procedures. The first synthetic procedure has involved an ultrasound assisted bromination in an aqueous medium using ionic liquid as a catalyst of the reaction, whereas in the second one an *in situ* formation of Br₂ via oxidation of HBr by H₂O₂ has been used. For comparison, the conventional bromination procedure was also used. The newly brominated compounds were characterized by appropriate analytical techniques. A detailed NMR spectroscopic analysis and quantum chemical calculations using Density Functional Theory (DFT) methods have been used to define the stereochemistry of the products. The results confirmed the physicochemical identity and similar yields of the products obtained by the three synthetic procedures employed, and reveal the co-existence of two diastereoisomeric forms of the newly synthesized products. The antibacterial and antifungal activities of the dibrominated amides were evaluated.

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1. Introduction

In the last years the comprising (C6–C3) skeleton cinnamic and hydroxycinnamic acid derivatives have gained particular attention due to their various biological activities such as antioxidant, anti-tyrosinase, hepatoprotective, anticancer, antimicrobial, etc. [1–8]. These cinnamic compounds belong to secondary plant metabolites and may occur either in free or conjugated forms.

Because of bacterial resistance, it is necessary to find a new alternative to conventional agents and cinnamic acids are considered as a value scaffold for different chemical modifications. The focus of some published extensive reviews [4,8] is directed especially towards cinnamic acids and their carboxylic derivatives (esters, amides, hydrazides) with antibacterial, antiviral and antifungal properties. However, to the best of our knowledge, little is

known about the influence of the side chain of the considered compounds on their antimicrobial activity. Only few reports have proposed that removal of the double bond by bromination of cinnamic acid and its derivatives cause remarkable enhancement of the antimicrobial activity [9,10].

It is well known that halogenation of an alkene stereoselectively leads to vicinal dihalides in *E*– π diastereomeric forms. Although this conventional process has a long history as a fundamental electrophilic addition reaction, the used molecular halogens are hazardous, toxic and corrosive. Furthermore, the halogenation is usually performed in chlorinated solvents, which are on the “environmental blacklist”. In the last few years, there has been rapid progress in the development of environmentally friendly synthetic methods for bromination of olefins using various types of oxidants and catalysts [11–16] or by employing bromine carrying agents [15,17–19]. On the other hand, ionic liquids have been described as benign solvents-catalysts systems for numerous reactions, including green halogen addition reactions to unsaturated compounds [17,18,20–22].

* Corresponding author.

E-mail address: mayabg2002@yahoo.com (M. Chochkova).

With an emphasis on the cinnamoyl derivatives, for example, ethyl cinnamate undergoes oxidative bromination by *in situ* generated bromine by the couple HBr/H₂O₂ [23] and another green sustainable bromination procedure proceeds in aqueous media and ionic liquids [23]. These emerge as important alternatives to the classical bromination for the synthesis of such kinds of dibromo compounds.

In this study, novel bromine derivatives of *N*-cinnamoylamino acid amides were obtained by development of alternative, environmentally friendly methods for bromination of *N*-phenylpropenoyl amino acid amides by implementation of simultaneously sonochemically activated procedures. Our interest was focused on synthetic approaches based on: i) ultrasound assisted bromination in an aqueous medium using ionic liquids and ii) *in situ* formation of Br₂ via oxidation of HBr by H₂O₂. An important part in the study was focused on the structure and stereochemical behavior of the new bromine derivatives of *N*-cinnamoylamino acid amides using detailed NMR spectroscopic analysis and quantum chemical calculations using DFT methods, for assessing their potential antimicrobial properties.

2. Experimental part

2.1. General information

All chemicals (reagent grade) used were purchased from Sigma Aldrich (FOT, Bulgaria). Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60F₂₅₄ plates (Merck, Germany). Visualization was accomplished with UV light and then with Ce-P-Mo reagent: 10 g Ce (SO₄)₂, 25 g H₃[P(Mo₃O₁₀)₄] × H₂O, 940 ml H₂O, 60 ml conc. H₂SO₄ solution followed by heating. The synthesized compounds were separated by preparative thin layer chromatography with silica gel 60 GF₂₅₄ (Merck, Bulgaria). The organic solvents were of analytical grade.

The test bacteria *Staphylococcus aureus* 209, (Gram-positive) and *Escherichia coli* WF+, (Gram-negative) were obtained from the Bulgarian Type Culture Collection. All bacteria were cultivated on Tryptic Soy Agar (Difco, USA). *Candida albicans* 562 grown on Sabouraud agar (Difco, USA) was used for screening antifungal activity. Melting points degree Celsius were determined with Stuart SMP 10 melting point apparatus and are uncorrected. Attenuated total reflectance infrared spectroscopy (ATR-IR) measurements were performed using Thermo Scientific Nicolet iS10 FT-IR device with ID5 ATR accessory (diamond crystal).

The NMR experiments were recorded on Bruker Avance III 600 or Bruker Avance III 400 spectrometer, operating at 600.13 and 400.15 MHz for protons respectively. The NMR spectra were acquired in deuterated chloroform (CDCl₃) at a temperature of 300 K. The solvent resonance peak at 7.24 ppm was used as a chemical shift reference. Standard 1D ¹H NMR experiments with 30° pulses, relaxation delay 2 s, 16 transients of a spectral width of 6600 Hz were collected into 64 K time domain points. Typical measuring conditions for the two dimensional (2D) ¹H/¹H COSY and NOESY (Nuclear Overhauser Effect Spectroscopy) spectra were: relaxation delay 2 s, 4 or 16 scans, a total 2 K data points in F2 and 256 data points in F1 over a spectral width of 7000 Hz. The NOESY experiments were acquired in phase-sensitive mode using the standard gradient selected pulse sequences with a relaxation delay of 2 s and mixing time of 400 ms. One dimensional (1D) NOESY spectra using selective refocusing with a shaped pulse [24–26] were recorded with relaxation delay of 4s, mixing time of 400 ms over a spectral width of 7000 Hz. 2D ¹H/¹³C HMCB experiments were carried out with a spectral width of ca 8000 Hz for ¹H and 30000 Hz for ¹³C, relaxation delay 1.5 s, Fourier transform (FT) size 2 K × 1 K. The UV spectra of the compounds were measured with an

“Agilent 8453” UV–vis spectrophotometer. Electrospray Ionisation (ESI) mass spectra were recorded on an Esquire 3000.

2.2. Chemistry

2.2.1. Method A

To the suspension of *N*-cinnamoylamino acid amides (0.3 mmol), 0.15 ml IL and water (0.5 ml) was added by syringe 0.45 ml Br₂. The resulting mixture was sonochemically stirred for 30 min. At the end of reaction, the mixture was evaporated to dryness, and further diluted with ethyl acetate. The organic phase was washed with brine and after evaporation of ethyl acetate the crude product was purified by crystallization with 96% C₂H₅OH/H₂O.

2.2.2. Method B

N-cinnamoyl amino acid amide (0.3 mmol) was dissolved in 2.3 ml ethanol and then 0.24 ml HBr was added. After 5 min stirring in ultrasound bath, to the mixture 0.24 ml (30%) H₂O₂ was added. The obtained dark yellow mixture was sonicated continuously for 0.5 h. The progress of the reaction was monitored by TLC (PE:EtOAc = 2:0.4). After completion of the reaction, the mixture was neutralized with a concentrated NaHCO₃ solution and then extracted with EtOAc. The organic solvent was evaporated *in vacuo* thereafter the crude solid was subjected to Preparative Thin Layer Chromatography (PTLC) and purified by recrystallization (96% ethanol/H₂O).

2.2.3. Method C

In a 25 ml flask *N*-cinnamoyl amino acid amide (0.3 mmol) was dissolved in 1 ml tetrachloromethane and 0.1 ml Br₂. The resulting reaction mixture was stirred at room temperature for 4 h and then evaporated *in vacuo*. The desired product was purified by PTLC using the system PE/EtOAc, and then recrystallized from 96% ethanol/H₂O as white crystals.

2.2.3.1. tert. Butyl-*N*-(α, β-dibromo-β-phenylpropanoyl)-alaninate (1). M.p. = 147–151 °C; **UV** (C₂H₅OH) λ max = 203 nm; **¹H NMR** (400 MHz, in ppm, solvent CDCl₃): δ 1.48 (d, 3H, J = 6.7 Hz, CHCH₃), 1.50 (s, 9H, C(CH₃)₃), 4.53 (dq, 1H, J = 6.8, 6.7 Hz, CHCH₃), 4.68 (isomer (R), d, 1H, J = 10.9 Hz, –CHBr), 4.72 (isomer (S), d, 1H, J = 10.9 Hz, CHBr), 5.44 (isomer (S), d, 1H, J = 10.9 Hz, CHBr), 5.45 (isomer (R), d, 1H, J = 10.9 Hz, CHBr), 6.55 (isomer (R), d, 1H, J = 6.8 Hz, NH), 6.58 (isomer (S), d, 1H, J = 6.8 Hz, NH), 7.30–7.45 (m, 5H, Ar–H). ESI-MS 434.1 [M+H]⁺, 436.1 [M+2 + H]⁺, 438.1 [M+4 + H]⁺. [IR (ATR)_umax] 3265.15, 1727.47, 1648.16, 1558.49, 1450.40, 1366.86, 1220.76, 1139.15, 693.95 cm^{–1}.

2.2.3.2. tert. Butyl-*N*-(α, β-dibromo-β-phenylpropanoyl)-phenylalaninate (2). M.p. = 145–148 °C; **UV** (C₂H₅OH) λ max = 204 nm; **¹H NMR** (400 MHz, in ppm, solvent CDCl₃): δ 1.42 (isomer (R), s, 9H, C(CH₃)₃), 1.43 (isomer (S), s, 9H, C(CH₃)₃), 3.14 (isomer (S), m, 2H, CH₂), 3.21 (isomer (R), dd, 2H, CH₂), 4.67 (isomer (R), d, 1H, J = 11.0 Hz, CHBr), 4.70 (isomer (S), d, 1H, J = 10.7 Hz, CHBr), 4.82 (bq, 1H, J = 6.9, 5.8 Hz, CHCH₂), 5.44 (isomer (S), d, 1H, J = 10.7 Hz, CHBr), 5.45 (isomer (R), d, 1H, J = 11.0 Hz, CHBr), 6.42 (isomer (R), d, 1H, J = 7.01 Hz, NH), 6.47 (isomer (S), d, 1H, J = 7.1 Hz, NH), 7.15–7.30 (m, 5H, Ar–H), 7.30–7.45 (m, 5H, Ar–H). ESI-MS 510.4 [M+H]⁺, 511.8 [M+2 + H]⁺, 513.8 [M+4 + H]⁺, 532.0 [M+Na]⁺, 533.9 [M+2 + Na]⁺, 535.9 [M+4 + Na]⁺, 555.0 [M+2Na]⁺, 557.0 [M+2+2Na]⁺, 559.0 [M+4+2Na]⁺. [IR (ATR)_umax] 3336.23, 1728.99, 1668.04, 1517.59, 1361.42, 1214.96, 1161.63, 1150.74, 694.36 cm^{–1}.

2.2.3.3. Methyl-*N*-(α, β-dibromo-β-phenylpropanoyl)-3-fluorophenylalaninate (3). M.p. = 137–138 °C; **UV** (C₂H₅OH) λ max = 205 nm; **¹H NMR** (400 MHz, in ppm, solvent CDCl₃): δ 3.20

(m, 2H, CH_2), 3.73 (s, 3H, OCH_3), 4.72 (isomer (R), d, 1H, $J = 10.9$ Hz, CHBr), 4.74 (isomer (S), d, 1H, $J = 10.9$ Hz, CHBr), 4.98 (m, 1H, CHCH_2), 5.44 (isomer (R), d, 1H, $J = 10.9$ Hz, CHBr), 5.46 (isomer (S), d, 1H, $J = 10.9$ Hz, CHBr), 6.52 (isomer (R), d, 1H, $J = 6.9$ Hz, NH), 6.50 (isomer (S), d, 1H, $J = 6.9$ Hz, NH), 6.70–7.10 (m, 4H, Ar–H), 7.10–7.45 (m, 5H, Ar–H). ESI-MS 486.0 $[\text{M}+\text{H}]^+$, 488.0 $[\text{M}+2+\text{H}]^+$, 490.2 $[\text{M}+4+\text{H}]^+$. [IR (ATR) $_{\text{u}_{\text{max}}}$] 3266.29, 1727.47, 1652.02, 1558.34, 1367.80, 1219.56, 1142.18, 694.24 cm^{-1} .

2.2.3.4. Methyl-*N*-(α , β -dibromo- β -phenylpropanoyl)-valinate (4**).** M.p. = 130–134 °C; **UV** ($\text{C}_2\text{H}_5\text{OH}$) $\lambda_{\text{max}} = 204$ nm; **^1H NMR** (400 MHz, in ppm, solvent CDCl_3): δ 0.98 (isomer (S), 6H, $\text{CH}(\text{CH}_3)_2$), 1.01 (isomer (R), 6H, $\text{CH}(\text{CH}_3)_2$), 2.16 (isomer (R), m, 1H, $\text{CH}(\text{CH}_3)_2$), 2.28 (isomer (S), m, 1H, $\text{CH}(\text{CH}_3)_2$), 3.35 (isomer (R), s, 3H, OCH_3), 3.79 (isomer (S), s, 3H, OCH_3), 4.68 (m, 1H, HNCH), 4.80 (isomer (R), d, 1H, $J = 11.0$ Hz, CHBr), 4.84 (isomer (S), d, 1H, $J = 11.0$ Hz, CHBr), 5.46 (d, 1H, $J = 11.0$ Hz, CHBr), 6.57 (isomer (R), d, 1H, $J = 8.2$ Hz, NH), 6.60 (isomer (S), d, 1H, $J = 8.2$ Hz, NH), 7.30–7.45 (m, 5H, Ar–H). ESI-MS 441.9 $[\text{M}+\text{Na}]^+$, 443.9 $[\text{M}+2+\text{Na}]^+$, 445.9 $[\text{M}+4+\text{Na}]^+$.

[IR (ATR) $_{\text{u}_{\text{max}}}$] 3265.97, 1727.11, 1651.99, 1541.36, 1368.13, 1219.49, 1142.19, 694.35 cm^{-1} .

3. Evaluation of antimicrobial activity

3.1. Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of all samples was determined by the microdilution method described by Andrews [27] by using 96-well standard microtiter plates. Briefly, 50 μl of two fold serial dilutions of examined samples were added to 50 μl microbial suspension adjusted to yield approximately 1.0×10^5 CFU ml^{-1} . MIC was encountered as the lowest concentration of examined sample that inhibits the visible microbial growth after 24 h incubation at 37 °C. For positive controls commercially available antibiotics tobramycin and ketoconazole were used. The solvent DMSO was tested as negative control. Three replicates were done for each compound.

3.2. Computational details

The geometries and normal mode vibrational frequencies of the compounds synthesized **1–4** were computed by DFT using Firefly QC package [28], which is partially based on the GAMESS (US) [29,30] source code. Geometry optimization of the two isomeric forms due to the different configuration at the asymmetric center in the molecule of compounds **1–4** was carried out by the hybrid B3LYP functional which combines the three-parameter exchange functional of Becke [31] with the LYP correlation one [32] using 6-31G(d,p) basis set. Vibration frequency calculations were performed numerically to obtain vibrational zero point and thermal energies and to validate that the found structures corresponded to the energy minima. The calculations were carried out without symmetry constraints by the gradient procedure. A gradient

convergence threshold of 1×10^{-4} hartree Bohr^{-1} was used.

To obtain more accurate energies, MP2/6-31G(d,p)//B3LYP/6-31G(d,p) single-point calculations were performed. To estimate the effect of the medium (chloroform) on the relative stabilities of the isomers of compounds **1–4**, we applied the polarizable continuum model (PCM) [33,34] as implemented in the Firefly QC package as single point calculations at the same level of theory, PCM//B3LYP/6-31G(d,p).

The proton and carbon chemical shieldings were calculated with the B3LYP functional and 6-31 + G(2d,p) basis set using the gauge-including atomic orbitals (GIAO) approach [35,36] and B3LYP/6-31G(d,p) optimized geometry. Solvent effect was accounted for by using the self-consistent reaction field method with the polarizable continuum model (PCM) formalism. The including of the solvent as dielectric in GIAO NMR calculations was used to estimate the effect of the medium chloroform (CDCl_3) on the chemical shifts of compounds **2** and **4**. In order to compare with the experimental data, the calculated absolute shieldings were transformed to chemical shifts using the reference compound tetramethylsilane (TMS): $\delta = \delta_{\text{calc}}(\text{TMS}) - \delta_{\text{calc}}$. Both $\delta_{\text{calc}}(\text{TMS})$ and δ_{calc} were evaluated with the same method and basis set. The NMR calculations were carried out using GAUSSIAN 09 program package [37].

4. Results and discussion

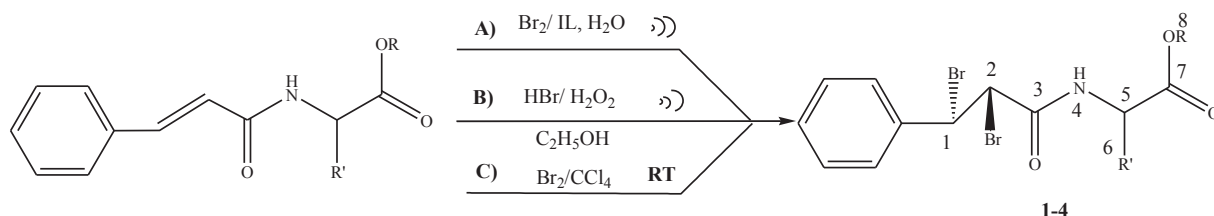
4.1. Synthesis

The preparation of the target α,β -dibromohydrocinnamoyl amino acid amides (**1–4**) was carried out as depicted in Scheme 1: by ultrasound assisted addition process, conducted in aqueous medium by ionic liquid (A); by ultrasound activated addition of Br_2 , generated by *in situ* oxidation of HBr by H_2O_2 (B); by conventional method (C).

The employed starting materials (*E*)-*N*-cinnamoyl amino acid amides were obtained using cinnamic acid and corresponding C-protected amino acids by means of EDC/HOBt coupling method, following the procedure reported in our previous work [6]. The reaction time, yield, atom economy and environmental factor E of the products for the applied synthetic methods are presented in Table 1.

The preparation of α,β -dibromohydrocinnamoyl amino acid amides by method A was followed by using of ionic liquid. Being 'green' recyclable alternatives to chlorinated solvent, herein, the commercially available ionic liquid (IL: 1-Butyl-3-methylimidazolium tetrafluoroborate ($[\text{bmim}][\text{BF}_4]$) was chosen. It is noteworthy, that ionic liquids have been known for their dual solvent-catalytic properties, and the bromination mechanism excludes the possibilities of obtaining by-products such as halohydrins or epoxides [21].

So, following the procedure adopted from Primerano et al. [21], we altered it by using additionally a new ultrasound-assisted bromination variant. In accordance with the established stoichiometric ratio between ionic liquid/water (1:3) [21] and considering the insolubility of *N*-cinnamoyl amino acid amides in reaction



Scheme 1. General scheme for side chain electrophilic bromination of *N*-cinnamoylamino acid amides.

Table 1

Yields, reaction time, atom economy and E-factor of the applied bromination methods.

Compounds	R'	R	Methods	Reaction time, h	Yields (%)	E-factor ^a	Atom Economy ^b (%)
1	CH ₃	C(CH ₃) ₃	A	1	60	20.3	65.5
			B	0.5	65	28.9	52.6
			C	4	57	25.7	99.5
2	–CH ₂ –C ₆ H ₅	C(CH ₃) ₃	A	1	40	26.6	69.1
			B	0.5	44	36.9	52.2
			C	4	40	31.8	99.6
3	–CH ₂ –C ₆ H ₄ (3-F)	CH ₃	A	1	39	28.5	68.0
			B	0.5	42	40.5	52.3
			C	4	37	36.1	99.6
4	–CH(CH ₃) ₂	CH ₃	A	1	45	28.3	86.0
			B	0.5	48	40.7	52.7
			C	4	45	33.9	99.5

^a Ideal is 0.^b Ideal is 100%; The environmental factor [38] includes solvents and excluded water.

mixture, herein the excess of bromine was used.

Method B is another green approach of Br₂ addition. The bromine was *in situ* generated using the couple HBr/hydrogen peroxide [23]. During this oxidation process, hydrogen peroxide is well-established as an environmentally-friendly reagent, due to formation of water as the only by-product [12].

In order to improve the rates of the bromination processes of *N*-cinnamoyl amino acid amides and reaction yields, method B was simultaneously sonochemically activated.

As it can be seen in Table 1, the yields obtained by methods A and B did not differ significantly from the conventional bromination (method C). Both green methods (A and B) have advantages over the classical bromination (method C) consisting in considerably shorter duration and gentle, non-toxic conditions of the procedures.

The newly obtained through the bromination amides were purified by preparative thin layer chromatography and further recrystallized as white crystals (96% ethanol/H₂O). The structures of the desired compounds were ascertained on the basis of melting points, UV, IR, NMR and mass spectral data.

4.2. Structure elucidation

In contrast to *N*-cinnamoyl amino acid amides, IR spectra of α,β -dibromohydrocinnamoyl amino acid amides have shown an absence of characteristic alkene peaks =C–H with medium absorbency in the range 3040–3000 cm^{−1} and aliphatic –CH=CH– stretching band at 1622 cm^{−1}. Moreover, the IR spectra of the newly obtained amides (1–4) show a sharp peak at 695 cm^{−1}, which is correlated with dibromide functionality.

Structural elucidation for both brominated and non-brominated *N*-cinnamoyl amino acid amides is greatly enhanced by positive ion ESI mass spectra. MS spectra recorded for α,β -dibromohydrocinnamoyl amino acid amides reveal the 1:2:1 pattern of molecular ions at m/z [M]⁺, [M+2]⁺ and [M+4]⁺, related to the presence of two bromine atoms, whereas these isotopic clusters are absent in *N*-hydroxycinnamoyl amides.

The structure of α,β -dibromohydrocinnamoyl amino acid amides prepared by three different methods was compared with the starting *N*-cinnamoyl amino acid amides by analysis of the 1D (¹H) and 2D (COSY, HMBC) NMR spectra. The resonance signals of the olefinic protons in all *N*-cinnamoyl amides studied appear as doublets at 7.5 (H1) and 6.4 (H2) ppm with vicinal spin-spin coupling constant (³J) around 16 Hz confirming *E*-configuration of the double bond. Characteristic upfield chemical shift of H1 (c.a. 5.5 ppm) and H2 (c.a. 4.6 ppm) resonances and decrease of their spin-spin coupling constant (³J_{H1/H2}–11 Hz) were observed in the ¹H NMR spectra of all brominated *N*-cinnamoyl amides (compounds

1–4) due to the electrophilic bromine addition reaction at the double bond of cinnamoyl residues. The chemical shift and spin-spin coupling constants of the protons in the ¹H NMR spectra of the novel bromine derivatives of *N*-cinnamoyl amino acid amides are presented in the Experimental part. The NMR results confirm the structural identity of the products obtained by the different synthetic procedures employed (Methods A–C). Representative ¹H NMR spectra of a bromine derivative of *N*-cinnamoyl amide (compound 2) prepared using different synthetic protocols are shown in Fig. 1 with assignment of the resonance signals.

In the ¹H NMR spectra of the products 1–4 a doubling of the resonance signals was observed; two sets of signals were clearly detected for H1, H2, H4 and methyl protons of –OCH₃ and –OC(CH₃)₃ groups in the spectra of all products. The relative quantitative distribution of the observed two forms was estimated by the integral intensity of the resonance signals of H4 and H2 in ¹H NMR spectra and was found to be 1: 1.1; 1: 1.5; 1: 1.3; 1:1.2 and 1:1.1 for 1–4, respectively. The analysis of ¹H NMR spectra recorded in the temperature interval between 290 and 320 K showed no changes in the spectral behavior of the compounds and quantitative distribution of the observed two forms. The NMR data suggest a co-existence of two isomeric forms of the synthesized new compounds most probably due to the different configuration at the C5 asymmetric center of the amide residues in the molecules.

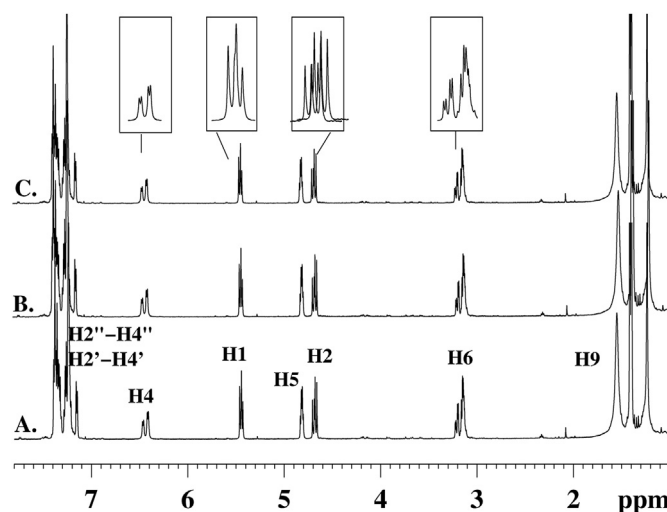


Fig. 1. 600 MHz ¹H NMR spectra of compound 2 obtained by methods A–C (Scheme 1) Selected spectral areas from the spectra are extended for better visualization of the signal splitting.

In order to obtain additional information for the structure of the compounds presented in Fig. 2, DFT calculations in the gas phase and in chloroform were performed. Full geometry optimization of the structures was carried out using B3LYP functional and 6-31G(d,p) basis set. According to the different configuration associated with the position of hydrogen atom at the asymmetric center of the molecules two isomeric forms (R and S) were considered. The free Gibbs energies and relative stabilities of the two isomers of compounds **1–4** was calculated at B3LYP/6-31G(d,p) and MP2/6-

31G(d,p)//B3LYP/6-31G(d,p) levels and are given in Table 2. It was found that the isomer R is more stable than S for all molecules. The energy difference between the two isomers is highest for **1** in gas phase and in solution. It decreases when the calculations were performed at MP2/6-31G(d,p)//B3LYP/6-31G(d,p) level of theory. The energy differences between the two isomers of compounds **1**, **2** and **3** are higher at B3LYP/6-31G(d,p) level than these at MP2 level. If we consider the molecules of **1–4** as embedded in chloroform (PCM//B3LYP/6-31G(d,p) level) the energy differences decrease

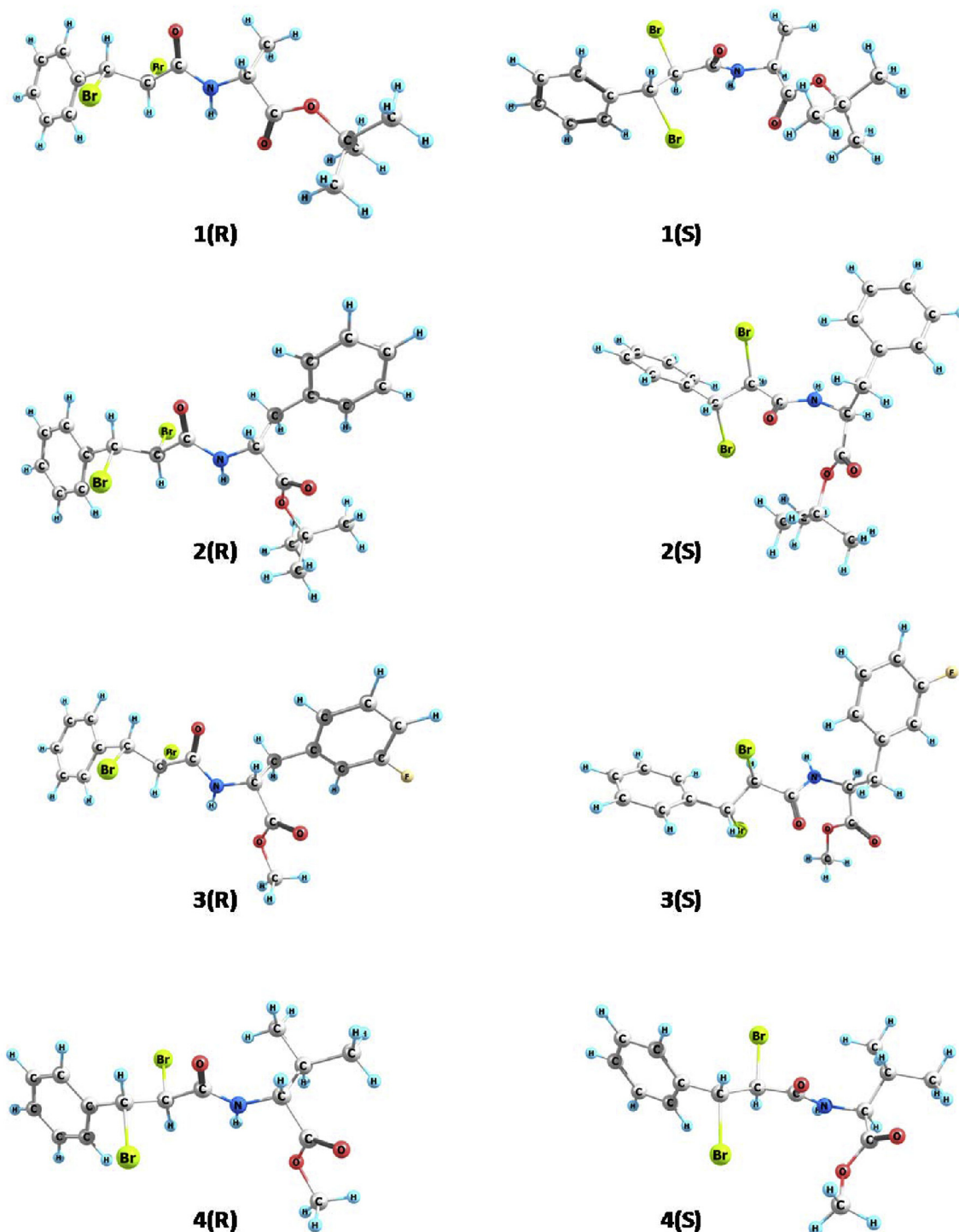


Fig. 2. B3LYP/6-31G(d,p) optimized structures of the isomers (R and S) of compounds **1–4**.

Table 2

Relative free energies (ΔG_{298}) in kcal mol⁻¹ for the two isomers of compounds **1–4** calculated at B3LYP/6-31G(d,p) level in gas phase and in chloroform. The single point calculations at MP2/6-31G(d,p) level are also given.

compounds	B3LYP/6-31G(d,p)		MP2/6-31G(d,p)//B3LYP/6-31G(d,p)
	gas phase	chloroform	gas phase
1(R)	0.00	0.00	0.00
1(S)	4.72	3.84	2.99
2(R)	0.00	0.00	0.00
2(S)	1.90	2.13	0.92
3(R)	0.00	0.00	0.00
3(S)	2.74	2.32	1.93
4(R)	0.00	0.00	0.00
4(S)	2.52	2.19	2.81

excepting compound **2**. Because of the low energy differences between the two isomers of molecules **1–4** (about 2 kcal mol⁻¹) the R and S structures should be presented in chloroform and only isomer S of compound **1** should have a low population.

4.3. Antimicrobial activity

The prevalence of the drug-resistance phenomenon forces many research groups towards the development of new highly active drugs. Since it was established that cinnamic acid is a promising molecule for antimicrobial activity, the removal of the double bond of its side chain through bromination was reported to cause a remarkable increase in growth inhibitory effect [9].

In this study our attention is on screening the antimicrobial activity of α,β -dibromohydrocinnamoyl amino acid amides against *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive), and one fungus *Candida albicans*. The results presented as minimal inhibitory concentrations (MICs) are compared with tobramycin and ketoconazole, used as reference standards for antibacterial and antifungal activity respectively (Table 2).

Regarding the obtained results for antibacterial activity, amongst the tested compounds, **1** and **4** showed marked activity against *S. aureus* (with MIC values of 156 $\mu\text{g ml}^{-1}$; Table 3). Moreover, compound **4** emerged as a highly active one, displaying the same growth inhibition (156 $\mu\text{g ml}^{-1}$) against Gram-negative species *E. coli*. For the pair of α,β -dibromohydrocinnamoyl amides of phenylalanine (compound **2**) and compound **3** it was observed that the *meta*-substituted fluorophenylalanine ring (compound **3**) improved the antibacterial activity (1.7 fold) in comparison with its non-substituted counterpart (compound **2**). In contrast, the whole group of amides can be considered not to be active against *C. albicans*.

The compounds (**1**) and (**4**) should be taken into consideration in terms of activities against both antibacterial strains *S. aureus* and *E. coli*.

According to the screening of antimicrobial activity of α,β -dibromohydrocinnamoyl amino acid amides the most active

compound against *S. aureus* and *E. coli* was compound **4** and the least active one was compound **2**. On the base of the results of antimicrobial activity of these compounds, we decided to deepen our attention to their structure.

A comprehensive structural characterization of the isomeric forms of **2** and **4** (Fig. 2) prepared by three different methods was achieved by analysis of the 1D (¹H and ¹³C) and 2D (COSY, HSQC, HMBC) NMR spectra. The NMR results confirmed the structural identity of the products obtained by the different synthetic procedures employed. Representative ¹H NMR spectra of compound **2** prepared using different synthetic protocols are shown in Fig. 1 with assignment of the resonance signals. The assignment of the resonance signals in ¹H and ¹³C NMR spectra of the compounds and spin-spin coupling constants measured are presented in Table 4.

The stereochemical features of the isomeric forms of **2** and **4** were established by means of the proton spin–spin coupling constants extracted by analysis of the ¹H NMR spectra and study of the Nuclear Overhauser Effect (NOE). The NOEs interactions observed in the 2D ¹H/¹H NOESY and 1D NOE spectra of **2** and **4** are presented schematically in Fig. 2. Fig. 3 shows representative 1D NOE spectra of **2** recorded in CDCl₃ solution at 300 K. The values of the vicinal spin-spin coupling constants (Table 4) and absence of NOE interaction between H1 and H2 protons confirm *trans* orientation of both Br atoms in the compounds **2** and **4**.

A detailed analysis of the data suggested the existence of two isomeric forms due to the different configuration at the C5 asymmetric center in the molecule of **2** and **4** (Fig. 4). The detected NOE interactions indicate R configuration in the more populated isomeric form and S configuration in the other. The stereochemical structures of both isomeric forms of the two compounds are presented in Fig. 4.

We present our GIAO NMR results considering chloroform solvation employing the polar-continuum model (Table 4). The predicted values of the chemical shifts for compounds **2** and **4** are unsatisfactory if the NMR calculations are performed in gas phase. More adequate results are obtained when NMR spectra is calculate in solution taking into account chloroform as solvent. The calculated ¹H and ¹³C NMR chemical shifts were found to be close to the experimental data obtained (Table 4).

The sensitivity of ¹³C NMR chemical shifts to the presence of polarization and diffuse functions in the basis set is a reason the 6-31 + G(2d,p) basis set to be employed [39]. Our theoretical results are in agreement with the ¹H and ¹³C NMR measurements in chloroform of compounds **2** and **4** (Table 4). The change of the substituents at C5 position influences the NMR spectra of the compounds. In the ¹³C NMR spectra of **2** and **4** the most significant chemical shift changes are observed for the three carbon nuclei – in CH₃, CH₂ and CH groups. The substituent change leads to more significant chemical shift alterations to the C9, C8 and C6 nuclei. In accordance with theoretical results the chemical shifts for C8 in **2**

Table 3

Antimicrobial activity of α,β -dibromohydrocinnamoyl amino acid amides expressed as minimal inhibitory concentrations MIC ($\mu\text{g.ml}^{-1}$).

Compound	MIC ($\mu\text{g.ml}^{-1}$)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
1	156	313	625
2	625	625	1250
3	375	375	NT
4	156	156	1250
Tobramycin	15.6	19.5	N.T
Ketoconazole	NT	NT	7.8

NT: not tested.

Table 4
GIAO ^1H and ^{13}C chemical shifts (in ppm) of the two isomers of compounds **2** and **4** calculated at B3LYP/6-31 + G(2d,p) level (in bold) and experimental data. J_{HH} are in Hz. The geometry is optimized at B3LYP/6-31G(d,p) level.

Compounds				
Nuclei	2		4	
	R $\delta^1\text{H}/\delta^{13}\text{C}$ (ppm), J_{HH} (Hz)	S $\delta^1\text{H}/\delta^{13}\text{C}$ (ppm), J_{HH} (Hz)	R $\delta^1\text{H}/\delta^{13}\text{C}$ (ppm), J_{HH} (Hz)	S $\delta^1\text{H}/\delta^{13}\text{C}$ (ppm), J_{HH} (Hz)
1	5.45 d/51.4 (11.0) 5.57/67.5	5.44 d/51.1 (10.7) 5.52/68.7	5.47 d/51.2 (11.0) 4.52/68.3	5.46 d/50.8 (11.0) 5.58/68.4
2	4.67 d/49.7 (11.0) 4.55/63.9	4.70 d/50.4 (10.7) 4.71/64.0	4.80 d/49.6 (11.0) 4.51/64.3	4.84 d/49.9 (11.0) 4.45/63.4
3	-/166.3 163.9	-/166.2 164.0	-/166.9 163.3	-/166.7 164.6
4	6.42 d/- (6.8) 6.38	6.47 d/- (7.1) 6.27	6.57 d/- (8.2) 6.13	6.60 d/- (8.2) 6.25
5	4.82/54.5 4.58/62.6	4.82/54.2 3.49/62.9	4.70 m/52.5 4.51/61.6	4.40 m/52.5 3.19/63.6
6a	3.21dd/38.2 (13.9, 5.1) 3.50/43.2	3.14 m/37.7 3.42/37.7	2.29 m/31.7 1.79/40.3	2.29 m/31.9 2.76/33.2
6b	3.14 m/38.2 2.54/43.2	3.14 m/37.7 3.14/37.7		
7	-/170.07 169.0	-/170.11 170.0	-/172.0 171.0	-/172.1 171.9
8	-/83.2 84.3	-/83.3 84.5	3.79 s/57.8 3.82/53.7	3.80 s/57.4 3.71/53.5
9	1.40 s/28.2 1.49/27.4	1.42 s/28.22 1.49/27.3	1.01 d/19.0 1.01/20.7	0.98 d/17.8 1.06/22.1
1'	-/138.3 135.4	-/138.2 139.0	-/138.7 136.7	138.4 137.1
2'-4'	7.40–7.30/130–127 8.15–7.67/127.8–126.2	7.40–7.30/130–127 7.70–7.40/128–126	7.45–7.30/130–128 7.75–7.58/127–126	7.45–7.30/130–128 7.70–7.50/128–125
1''	-/136.0 136.21	-/135.8 139.0		
2''–4''	7.30–7.10/130–127 7.93–7.14/127–124	7.30–7.10/130–127 7.60–7.50/128–124		

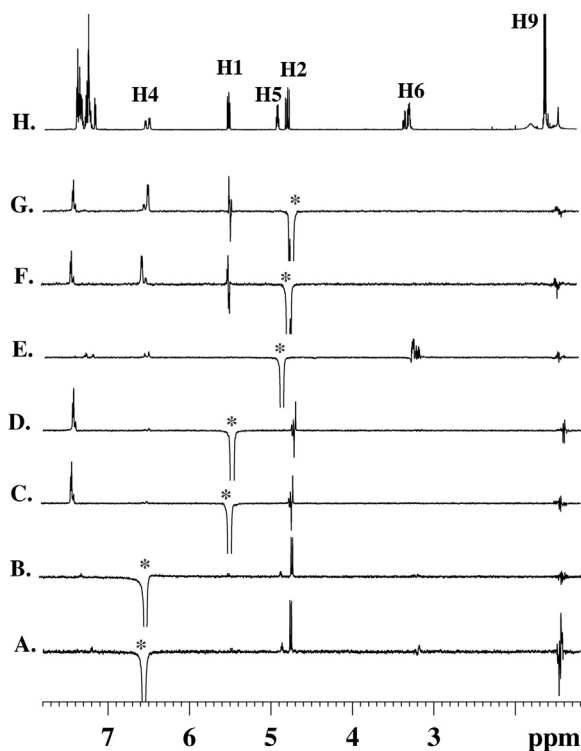


Fig. 3. 600 MHz selective 1D NOESY spectra of **2**. The irradiated resonance signals are marked.

are 84.3 ppm for R isomer and 84.5 ppm for isomer S. This value becomes 53.7 ppm (R) and 53.5 ppm (S) in **4**. There are insignificant chemical shift alterations to C5 of **4** in comparison to **2**. When we consider the ^{13}C NMR spectrum of **2** the change of asymmetric center configuration leads to chemical shift alteration only to C6, 43.2 ppm in the R structure and 37.7 ppm in the S one. The change in chemical shifts of C6 is most strongly expressed in the spectrum of **4** – from 40.3 ppm in R to 33.2 ppm in S.

Some changes are observed in calculated ^1H NMR spectra of both above-mentioned compounds. The alterations are related to the position of the H atom at the asymmetric center (Table 4). The computed chemical shifts of H5 in isomer R of compound **2** and **4** are closed – 4.58 ppm and 4.51 ppm, respectively. However, a significant difference between the chemical shifts of H5 in respect to the two isomeric forms of each compound was detected. The results are indicative of changes in the configuration of the asymmetric center at position C5 in compounds **2** and **4**. These configuration changes could explain the significant difference in the chemical shifts of H5 in the two isomeric forms detected in the ^1H NMR spectra of the compounds (Table 4). The calculated chemical shifts of H5 in compound **2** were determined to be 4.58 and 3.49 ppm for the R- and S-isomeric forms, respectively. The configuration change at the C5 asymmetric center in compound **2** leads to the ^1H NMR shift of H5 from 4.58 ppm in R to 3.49 ppm in S. For compound **4**, the resonance frequency of H5 shifts from 4.51 ppm in the R-isomeric form to 3.19 ppm in the S-isomeric form. Significant chemical shift changes were also observed for the methylene and methane protons at position C6 of compounds **2** and **4**, being in a close proximity to the asymmetric center C5.

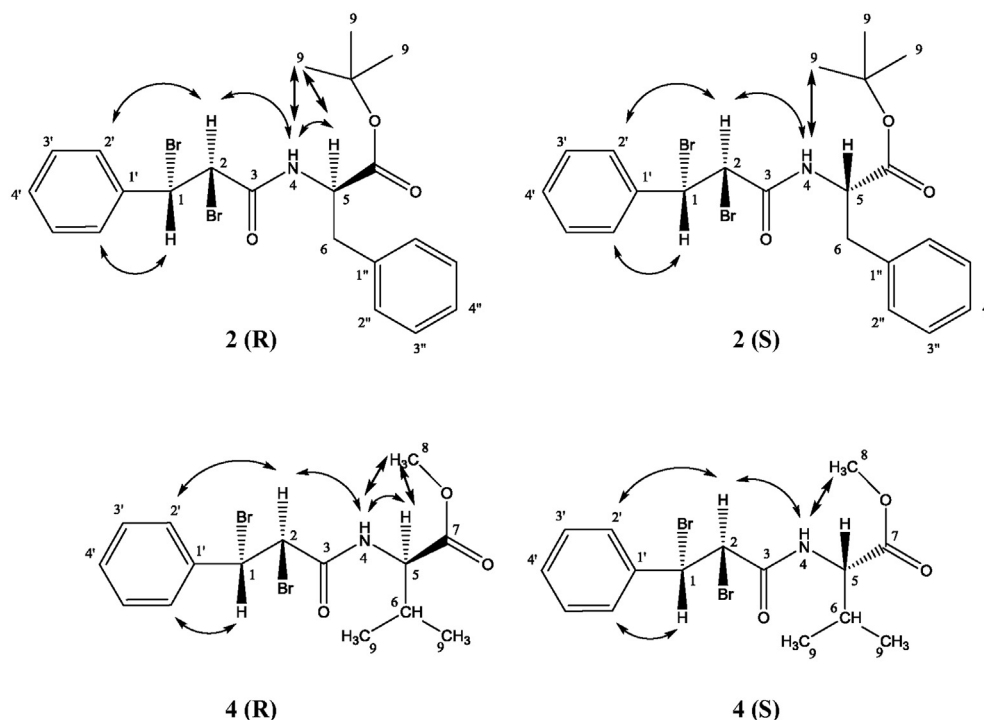


Fig. 4. Structure of the two isomeric forms of compounds 2 and 4. The main NOE are marked by arrows.

5. Conclusions

Four new α,β -dibromohydrocinnamoyl amino acid amides were readily synthesized following two green sonochemically modified and a conventional brominating procedures. The yields obtained by the green sonochemically modified methods A and B did not differ significantly from the conventional brominating. The applied green methods (A and B) have advantages over the classical brominating consisting in considerably shorter durations and gentle, non-toxic conditions of the procedures.

The antimicrobial activities of the compounds studied reveal that compounds 1 and 4 could be candidates for further studies of antibacterial activity.

DFT B3LYP/6-31G(d,p) and MP2/6-31G(d,p)//B3LYP/6-31G(d,p) calculations in gas phase and in chloroform were performed to obtain information about the structure of the compounds synthesized. We considered two possible isomeric forms (R and S) of the compounds due to the different configuration at the asymmetric center in the molecule. According to the relative stabilities of the two isomers of compounds 1–4 isomer R is the most stable structure in all molecules. The energy differences between R and S in chloroform are about 2 kcal mol^{-1} and this suggests that the two configurations should be present in this solution. The exception is compound 1 where structure R is more stable than S by $3.84 \text{ kcal mol}^{-1}$ and should be in a small amount in the solution.

The presence of two isomeric forms in chloroform was confirmed by theoretical and experimental ^1H NMR spectra as well as by study of the Nuclear Overhauser Effect (NOE) of compounds 2 and 4.

Conflict of interest

The authors declare no conflict of interest.

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