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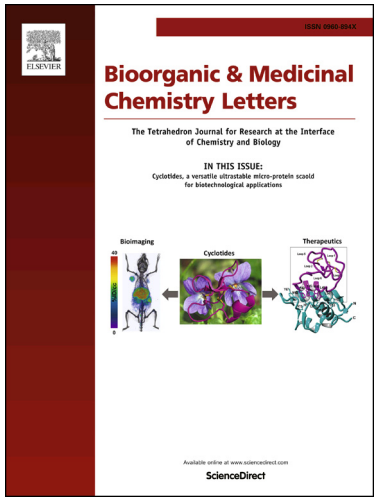

Towards smart biocide-free anti-biofilm strategies:Click-based synthesis of cinnamide analogues as anti-biofilm compounds against marine bacteria.

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Towards smart biocide-free anti-biofilm strategies : Click-based synthesis of cinnamide analogues as anti-biofilm compounds against marine bacteria.

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Biofilms results in the colonization process of soft or hard, artificial or living substrata by microorganisms which attach to surfaces and bind to one another.[1] Although the control of the development of planktonic bacteria communities is well known and mastered, bacteria within a biofilm are much more resistant to antibiotic or biocide treatment (up to 1000-fold increased resistance). To fight biofilms, the massive use of such compounds has led to major problems in human health by developing high degrees of resistance in bacterial communities as well as economic, environmental and toxicological issues leading to establishment of strict regulations.[2-5] In this context, targeting the formation of bacterial biofilms in a non-toxic way is of great interest in view of a rational use of antibiotics and/or biocides and the development of original non-toxic biofilm inhibitors should have the potential to be used in a preventive treatment of a wide diversity of industrial and medical surfaces. Moreover, the development of such solutions must remain competitive and must enable low-cost molecules to be placed on the market. For such a challenge, some of the anti-biofilm strategies that are pursued todays consist in studying structure-activity relationships (SAR) of simple secondary metabolites from marine organisms such as sponges or soft corals in view of discovering new specific and non-toxic anti-biofilm leads which should be used as potential adjuvant for antibiotherapies or friendly environmentally biocides. [6-8] In this field, we are developing an efficient and simple approach based on the bioisosteric replacement of natural frameworks by a 1,2,3-triazolic ring to allow SAR studies in the field of antifouling fight, and we have previously designed with success isonaamine A, bromotyramines and hemibastadins analogues (fig. 1).[9-11] For this purpose, ‘click chemistry” by mean of copper-catalyzed azide-alkyne cycloaddition was used as a highly efficient approach, offering substantial advantages, since it is tolerant to multiple functional groups.[12-13] In addition a large variety of terminal alkyne is available as building blocks, while azides may easily be accessed from common precursors, such as halides and amines, using standard literature procedures.

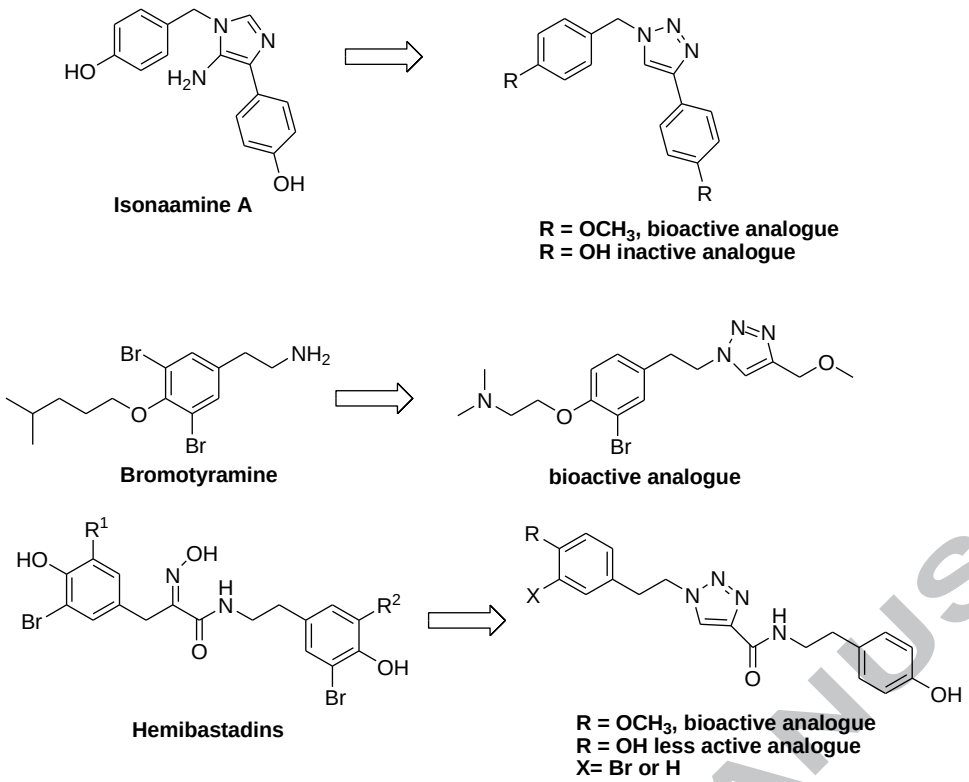


Figure 1 : click-based design of natural product analogues [9-11]

Pursuing these investigations in the field of antibiofilm compounds, we are interested in the advanced SAR studies of tyramine derivatives and we wish to investigate some more simple frameworks which could be accessible in a two-steps process from simple commercial tyramines and anilines. To assume such a challenge, we focused on the design of original analogues of cinnamoylphenethylamine family; this class of natural products are found in over 30 plant families. The most ubiquitous are p-coumaroyltyramine and feruloyltyramine. Nevertheless, the cinnamic acid and phenethylamine derivatives are of great interest due to their plethora of associated biological activities such antibacterial and antimicrobial[14-16] compounds and more especially as anti-biofilm compounds.[17] In this work we planified a bioisosteric replacement of the double bond by a 1,2,3-triazole ring (fig. 2). Such bioisosteric replacements of double bonds have already been investigated with success to design analogues of combrestatin and resveratrol analogues which exhibited enhanced antitumoral potential.[18-19] In this way the cinnamic acid moiety is modified. Furthermore, considering our previous results which showed that methoxy derivatives were more efficient than the hydroxy ones,[11] we decided to restrict the study to methoxy anilines **1a** and **1b** as starting materials.

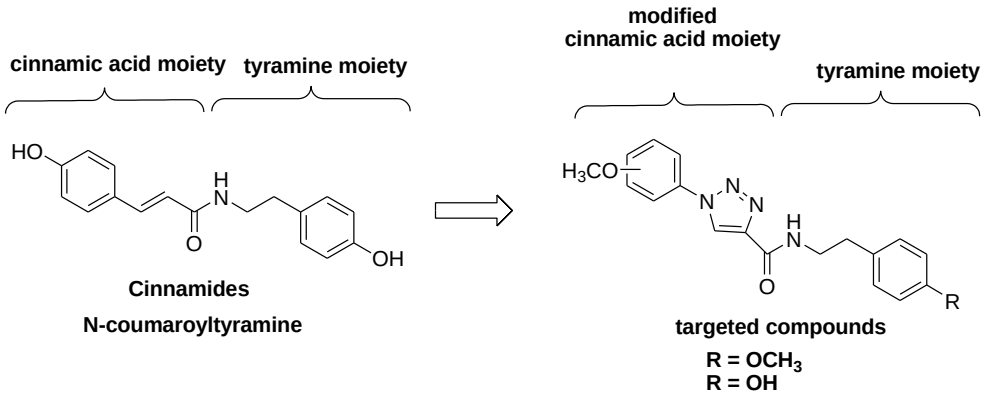
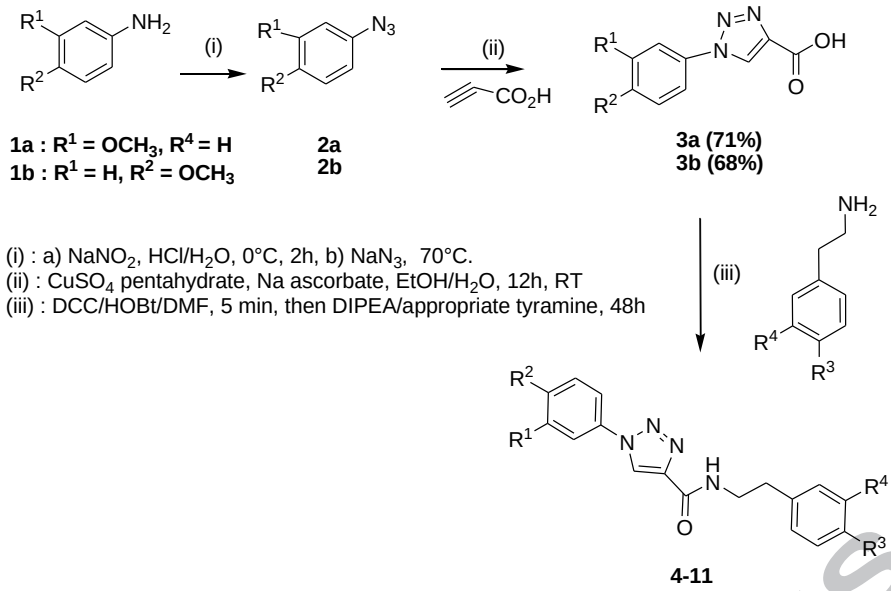


Figure 2. Structure of the targeted compounds

The first step of this work was the preparation of intermediary carboxylic acids (**3a, 3b**) (scheme 1). These compounds were obtained in excellent yield in two steps. Treatment of anilines (**1a, 1b**) with sodium nitrite followed by sodium azide in dimethylformamide afforded the corresponding crude azides (**2a, 2b**) which were used without further purification. Synthesis of the targeted carboxylic acids (**3a, 3b**) was then achieved by performing the copper(I)-catalyzed 1,3-dipolar cycloaddition of the organic azides with propargylic acid resulting in the formation of 1,2,3-triazoles.[20] In general, these reactions usually proceed to completion in 6–36 h at room temperature in water with a variety of organic co-solvents, such as *tert*-butanol, ethanol, DMF, DMSO, THF, or CH_3CN.[12,21] Ethanol was chosen rather than DMF to allow an easier workup and a better purity of products as described in our previous work.[9] In practice, propargylic acid was added to a solution of appropriate azide (**2a, 2b**), $CuSO_4$/sodium ascorbate in a water/ethanol mixture (50/50) and the reaction time was optimized at 12 hours at room temperature. Further access to the different cinnamides analogues **4-11**, was allowed by a peptide coupling step using DCC/HOBt methodology.[22,23] All amides were obtained in good yields from starting azido compounds (table 1).



Scheme 1 : Synthesis of targeted cinnamide analogues from methoxyanilines **1a, 1b**

Table 1 : selected 1,4-disubstituted 1,2,3-triazoles

R^1	R^2	R^3	R^4	Cpd (Yield)
OCH_3	H	OH	H	**4** (50%)
OCH_3	H	H	OCH_3	**5** (43%)
OCH_3	H	OCH_3	H	**6** (78%)
OCH_3	H	OCH_3	OCH_3	**7** (79%)
H	OCH_3	OH	H	**8** (64%)
H	OCH_3	H	OCH_3	**9** (50%)
H	OCH_3	OCH_3	H	**10** (66%)
H	OCH_3	OCH_3	OCH_3	**11** (39%)

In order to assess anti-biofilm activity of these compounds against representative Gram-negative bacterial biofilms, three strains were chosen for their capacity to form biofilms: *Pseudoalteromonas lipolytica* (TC8), *Pseudoalteromonas ulvae* (TC14) and a *Paracoccus* sp. strain (4M6),[24] by using our previous method adapted from Leroy et al. using the specific fluorophore Syto®61.[25,26,27] Results of this screen are outlined in Table 2 as effective concentrations to inhibit 50% of the bacterial adhesion (expressed as EC_{50}). First, we can note that response of the three strains are different and that TC14 is more sensitive to this class of compounds than 4M6 and TC8 strains. The second point concerns the natural *N*-coumaroyltyramine which does not exhibit significant efficiency. Then, the results highlighted

some preliminary SAR information. Compounds possessing a 4-hydroxy group on the tyramine moiety (**4** and **8**) are not active (EC_{50}> 100 μM) confirming our hypothesis and previous results which showed that hydroxy groups are not beneficial for the activity, while all methoxy derivatives are active. We can also remark that the position of the methoxy group on the tyramine moiety affects the activity and that the 4-methoxy derivatives **6** and **10** gave rise to the best anti-biofilm, in the same order of magnitude of ampicillin against TC14 (11.4 μM and 11.3 μM respectively *vs* 9.3 for ampicillin). In the same way, addition of a supplementary methoxy group (compounds **7** and **11**) results in reduction of effectiveness, especially in the case of compound **7** (73.7 μM). Otherwise, no precisions concerning the SAR on the modified cinnamic moiety could be highlighted at this stage since the position of the methoxy group on the 3-position or the 4-position of the aromatic ring does not affect the activity (**4** *vs* **8**, **5** *vs* **9**, **6** *vs* **10**), while we can observe a modulation of activity in the case of **7** and **11** (3,4-dimethoxy on the tyramine moiety).

Table 2 : biological screening against bacteria biofilms

compounds	**4M6*** **(EC_{50}, μM)**	**TC8*** **(EC_{50}, μM)**	**TC14*** **(EC_{50}, μM)**
4	>300	275.5 ± 25	204.3 ± 26.0
5	226.4 ± 16	>300	80.6 ± 22.0
6	215.5 ± 25	>300	**11.4 ± 1.0**
7	153.5 ± 9	>300	73.7 ± 17.0
8	219.5 ± 12	>300	115.3 ± 25.0
9	213.6 ± 23	142.3±0	**52.3 ± 22.0**
10	259.6 ± 66	>300	**11.3 ± 7.0**
11	162.5 ± 5	>300	**54.6 ± 11.0**
N-coumaroyltyramine	>300	>300	75.5 ± 20
Ampicillin	144.1 ± 0.6	17.9 ± 1.0	9.3 ± 1.0

***4M6** : *Paracoccus* sp. **TC8** : *Pseudoalteromonas lipolytica*, **TC14** : *Pseudoalteromonas ulvae*

The further step was finally to investigate if these compounds exhibited a specific anti-biofilm activity or if this observation was simply related to a general toxic effect on the bacteria. For this purpose, both growth inhibition and viability assay were performed.[28] We have already shown that the antibiofilm activity of ampicillin is directly connected to an antibacterial and general toxic effect on these different strains (TC8, TC14, 4M6),[10,11] but in contrast, the results presented in figure 3 and 4 showed that when compared to untreated samples, the compounds **6, 9, 10, 11** exhibit no effect on the bacterial growth of TC14 strain (fig. 3).

Figure 3. Effect on TC14 growth of compounds **6, 9-11** at concentrations of 100 µM

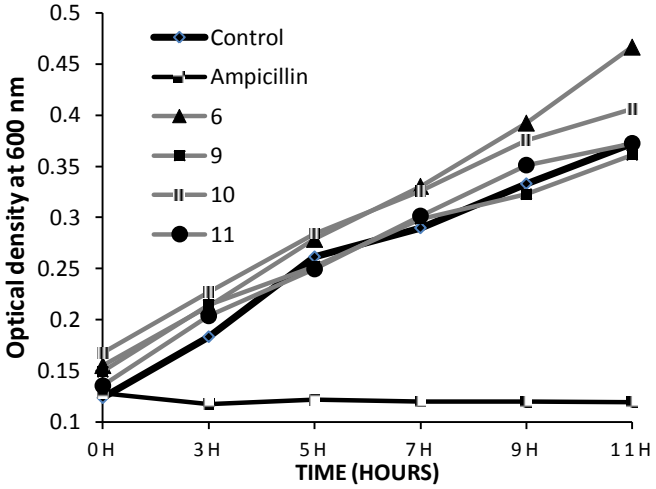
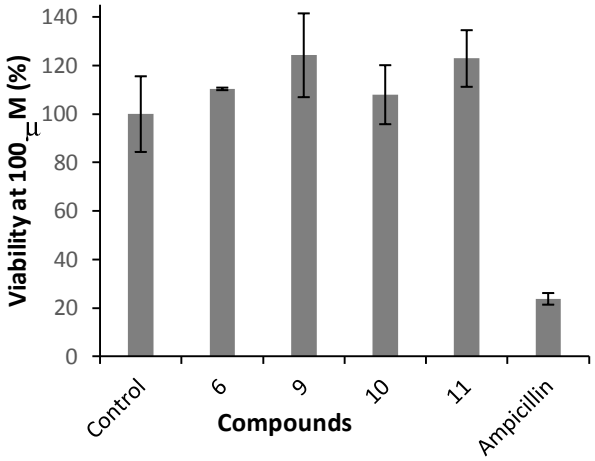


Figure 4. Effect on TC14 viability of compounds **6, 9-11** at concentrations of 100 µM.



For viability, the same methodology used for antiadhesion assay with Syto®61 was applied using resazurin test (fig. 4). Results showed that, when compared to ampicillin, none of the tested compounds (**6, 10-11**) exhibited any effect on the viability of the bacteria clarifying that these compounds were not lethal at 100 µM to the bacteria and suggested that the anti-biofilm activities of such compounds were not connected to antibacterial effect.

In conclusion, we have designed original analogues of cinnamides (*N*-coumaroyltyramine) based on the bioisosteric replacement of the cinnamic double dond. Interest of the work resides in the simple two-steps synthetic approach (copper(I)-catalyzed 1,3-dipolar cycloaddition followed by a peptide coupling step). This class of compounds exhibits specific anti-biofilm activity against a gram negative strain (*Pseudoalteromonas ulvae*,TC14). Futhermore, the low toxicity of the more potent derivatives when compared to ampicillin, should allow their rational use as co-antibiotic and/or co-biocides in view of eradication of biofilms in various applications such as biomedical materials, antifouling coatings, water pipelines network. Further studies are now needed to define more precisely the structure-activity relationships and their effect as potential adjuvents.

Acknowledgments

The *Paracoccus* sp. strain 4M6 was provided by the LBCM (Université de Bretagne Sud).

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20- **Typical method for preparation of compounds 3a, 3b**: to a stirred solution of 1g of methoxyaniline (**1a, 1b**) in H_2O/HCl (50/50, 15mL / 15 mL) was added 0.8g (11.55 mmol.) of $NaNO_2$. The whole was stirred at 0°C for 2h, and then NaN_3 0.78g (11.55 mmol.) were added. The resulting solution was refluxed for 3h. After extraction with dichloromethane, 100 mg of the resulting crude azide was dissolved in a solution of H_2O/EtOH (1.5 mL/1.5 mL) containing $CuSO_4$ pentahydrate (0.3 eq.), propiolic acid (1.5 eq.) and sodium ascorbate (0.4 eq.). The resulting mixture was stirred 12 hours at RT. A saturated solution of Na_2CO_3 was added and the resulting solution extracted 3 times with ethyl acetate. The organic layers was then dried over Na_2SO_4 and evaporated to give the crude triazoles which were purified by flash chromatography on silica gel eluted with hexane/ethyl acetate (80/20).

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22- **Typical method for preparation of compounds 4-11 :**[23] To a stirred solution of the acid **3a or 3b** (250 mg, 1.28 mmol) in anhydrous DMF, were added dicyclohexylcarbodiimide (1,2eq) and Hydroxybenzotriazole (1.2eq). The mixture was strirred at room temperature under nitrogen for 5 min, then appropriate tyramine (1eq) and diisopropylethylamine (100 uL) were added. The reaction was stirred at room temperature for 48 hours. A saturated NaCl solution was added and the organic layer extracted three times with CH_2Cl_2. The solvent was removed in vacuo and the residue purified by flash chromatography (SiO_2) using CH_2Cl_2/MeOH as eluent to yield compounds **4-11**.

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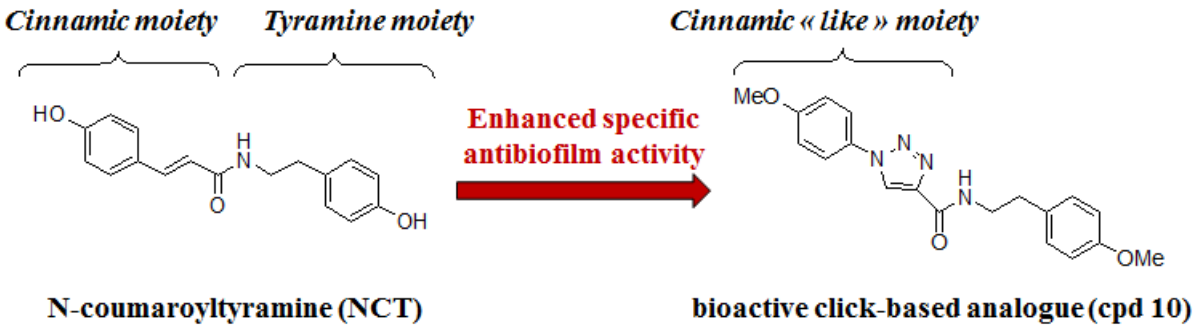
27- *Bacterial adhesion assays* (adapted from Leroy et al.[26]). *Pseudoalteromonas lipolytica* (TC8) was grown on VNSS (Vaatanen Nine Salt solution) at 20°C and sampled at the stationary phase. After centrifugation, cells were suspended in sterile artificial sea water (ASW) until an optical density of 0.2-0.4 at 600 nm was achieved. 200 µL of ASW was inoculated on the border-row wells of the 96-well microtiter plates (sterile black polystyrene NUNC), and 100 µL of the bacterial suspension on other wells using an eight-channel pipette. 100 µL of diluted standard biocide (Seanine) and purified molecules were added in the latter wells. All the concentrations were tested in triplicates. 100 µL of ASW was added in six wells to constitute the bacterial adhesion control. After 15 h at 20°C, the non-adhered bacteria were eliminated by three successive washes (36 g/L of sterile NaCl solution). The adhered bacteria were stained 10 min at room temperature by adding 200 µL of 40 µmol/L Syto® 61 (Invitrogen, USA). The excess stain was removed by two washes (36 g/L NaCl solution). The Syto® 61 was then solubilized in 200 µL of 36 g/L NaCl solution and fluorescence was measured (kexc = 615 nm, kem = 670 nm) using an Infinit 200 micro-plate fluorescence reader (TECAN, Lyon, France). The dose-response curves fitting and the determination of the EC50 for each molecule were achieved using GraphPad Prism software.

28- *Antibacterial assays*. Bacterial strains were grown on VNSS at 20°C under shaking conditions (120 rpm) and collected during the exponential phase. After centrifugation, cells were suspended

in sterile VNSS (OD600 nm = 0.1). 180 µL at different concentrations for each tested compounds (standard biocides, natural or natural-derived products) were added in four wells of the microtiter plates (sterile transparent PS; Nunc, Fisher Scientific). All the concentrations were tested in triplicate and the fourth well was filled for control. The maximum percentage of solvent used for the dilution of biocides was also tested in triplicate as additional control. For the growth inhibition control, 180 µL of VNSS was added in six wells. Then 20 µL of the bacterial suspension was inoculated on all the wells except the border-row wells and all the wells were filled out to 200 µL with VNSS. Turbidity (OD600 nm) was measured every hour during 6 h. Then, resazurin (50 µM) was added in all the wells, and fluorescence was measured after 2h to quantify the percent of bacterial viability. The same methodology used with SYTO 61 was applied to calculate a percent of viability after resazurin staining.

29- *Spectra data of the representative compound **10*** : N-(4-Methoxyphenethyl)-1-(-4-methoxyphenyl)-1*H*-1,2,3-triazole-4-carboxamide ^{1}H NMR (400 MHz, $CDCl_3$) δ : 2.89 (t, 2H, J = 7.1 Hz), 3.71 (m, 2H), 3.79 (s, 3H), 3.87 (s, 3H), 6.86 (d, 2H, J = 8.6 Hz), 7.04 (d, 2H, J = 9.0Hz), 7.17 (d, 2H, J = 8.6 Hz), 7.63 (d, 2H, J = 9.0 Hz), 8.39 (s, 1H), ^{13}C NMR (100 MHz, CD_3OD) δ : 34.9, 40.6, 55.2, 55.6, 114.1 (2C), 114.9 (2C), 122.3 (2C), 123.4, 129.7 (2C), 130.6, 143.9, 157.5, 158.3, 159.9, 160.2.

Graphical abstract



N-coumaroyltyramine (NCT) **bioactive click-based analogue (cpd 10)**