Multi-gram preparation of cinnamoyl tryptamines as skin whitening agents through a chemo-enzymatic flow process

David Roura Padrosa[a], Martina L. Contente[b]*

[a] Department of Chemistry, Biochemistry and Pharmaceutical Sciences, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland;

[b] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via Celoria 2, 20133 Milan, Italy

Abstract

A 2-step flow-based chemo-enzymatic synthesis of selected cinnamoyl tryptamines as potential cosmetic ingredients has been developed. A first reaction catalyzed by immobilized $Pd(OAc)_2$ gave the acyl donors employed as starting material in the second step, with very good yields (67-70%) and rapid reaction times (30 min). A second bioreactor made of imm-MsAcT, a glyoxyl-agarose immobilized acyl transferase from *Mycobacterium smegmatis*, was employed for the fast and efficient preparation of the desired amides (58-70% m.c., 15 min). In-line work-up allowing for the recovery and reuse of the unreacted substrates was added downstream the process, enhancing its automation. The combination of flow facilities, high substrate-to-catalyst ratio and closed-loop strategies make this methodology a sustainable and cost-effective procedure. Computational studies were carried out to provide insights into the enzymatic active site and substrate recognition.

Keywords

Flow-biocatalysis; enzyme immobilization; MsAcT; cinnamoyl tryptamines, skin whitening agents, chemo-enzymatic flow-synthesis.

Introduction

Continuous processing of fine chemicals such as pharmaceuticals, nutraceuticals and cosmetics is rapidly expanding at industrial level.[1–3] At the same time, biocatalysis is considered one of the key techniques for a greener way to operate in chemistry,[4,5] and the combination of biocatalysts with continuous processes has lately come up as a powerful tool to enhance process selectivity, productivity and sustainability.[6–8] In this context, continuous chemo-enzymatic syntheses provide innovative routes for the preparation of valuable compounds as they merge together the advantages of flow reactors (*i.e.,* better parameter control, higher mass and heat transfer,

modularity), the flexibility of chemical transformations and the selectivity of biocatalysts (*i.e.,* chemo-, regio-, and stereoselctivity).[9–11] We recently exploited an immobilized acyl transferase from *Mycobacterium smegmatis* (MsAcT) as a versatile catalyst for the flow-based preparation of a variety of different esters and amides as food related compounds,[12,13] nutraceuticals,[14] and APIs (active pharmaceutical ingredients)[15] both in water medium and organic solvents. In the present work we applied the catalytic power of MsAcT to the multi-gram preparation of cinnamoyl tryptamines, which have been recently proposed as potential natural cosmetics.[16] Due to consumer demands and environmental limitations, the cosmetic industry is in continuous search for new ingredients, better if obtained from natural sources, with environmentally-friendly connotations and less toxic properties.[16] The ability of cinnamoyl tryptamines to inhibit melanin production while showing low cellular toxicity makes them good candidates as skin-whitening agents.[17,18] Although plants are good natural sources, these molecules are typically secondary metabolites accumulated in very low amounts. Moreover, their extraction and purification are cost and energy consuming, and due to the high quantity of solvents usually employed, this practice cannot be considered sustainable.[19] An improvement was achieved with their microbial production through an engineered strain of *E. coli,*[20] but despite the advantages in terms of production and purification, cinnamoyl tryptamine and serotonin were only obtained in micromolar concentrations. On the other hand, common chemical procedures for the preparation of these compounds usually require anhydrous reaction environment, stoichiometric acylating agents as well as harsh reaction conditions, generating a significant amount of waste.[21] The flow chemo-enzymatic strategy here proposed allowed for the obtainment of the desired amides in very good yields with minimal consumption of organic solvents. The use of pure toluene for the enzymatic reaction demonstrated high biocatalyst stability and good solubility of the apolar substrates thus allowing for more productive methodologies. Where possible, closed-loop systems have been applied for the generation of low environmental impact reactions and waste reduction.

Results and Discussion

In order to enhance the stability of MsAcT, it was immobilized onto glyoxyl-agarose beads (imm-MsAcT), which was selected as best support after an in-depth immobilization study previously performed by us.[14] This hydrophilic carrier allowed not only for a high recovered enzymatic activity (73%) with low biocatalyst loading (1 mg/g_{resin}) but also the easy catalyst incorporation in flow chemistry reactors (packed bed reactor, PBR), thus guaranteeing controlled fluid dynamics and

acceptable residence times.[22] It was previously already highlighted that vinyl esters are more efficient acyl donors than the ethyl counterparts[14,23,24] for MsAcT-mediated catalysis, so activated vinyl cinnamates were firstly prepared in continuous mode (Fig. 1). Exploiting a transvinylation reaction through immobilized $Pd(OAc)_2$, cinnamic acids **1a** and **1b** were efficiently transformed into the corresponding esters (75% and 70% m.c. respectively) in 30 min of residence time. Batch reactions previously performed gave the same products with low yields and prolonged reaction times (40-45%, 24 h). Filtration steps necessary in batch mode to remove traces of $Pd(OAc)_2$, which is lost during the reaction work up, were here avoided and the process related costs dramatically reduced as catalyst was easily recycled. Through a "*catch-and-release*" protocol by an ion exchange resin (A 21) the unreacted acids were also recovered with the possibility to reuse them into the system. After column chromatography **2a** and **2b** were employed as acyl donors in MsAcT biocatalyzed flow reactions.

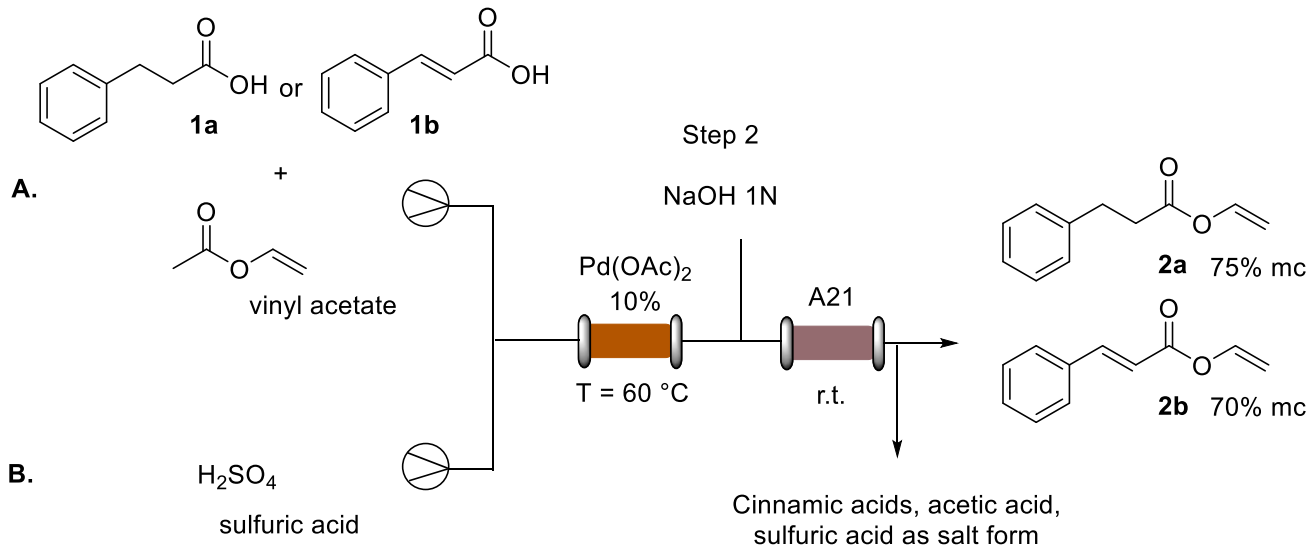


Figure. 1 Flow-based preparation of vinyl cinnamates. **A.** Solution of hydrocinnamic acid or *trans*-cinnamic acid (10 mmol) and vinyl acetate (160 mmol) in THF. **B.** Solution of H_2SO_4 (20% *w/w*) in THF. R.t. = room temperature, m.c. = molar conversion.

Due to the poor water solubility of **2a** and **2b** even after the addition of co-solvents (*e.g.,* DMSO), and the high stability of imm-MsAcT in organic solvents,[15,25] the second reaction was performed in pure toluene (Fig. 2). Using a 1.2 mL bioreactor, different residence times were evaluated (*i.e.,* 15 min, 30 min and 60 min) monitoring the reaction outcome through HPLC. Due to the high local

concentration of the biocatalyst and the efficient mixing the desired amides **4a**, **4b**, **4d**, and **4e** were obtained with good yields (58-70%) in 15 min of residence time. Notably, batch biotransformations gave lower conversions (25-32%) in much longer reaction times (24 h). Moreover, no hydrolysis side-reaction of the acyl donors, typical of batch mode was observed. To test the productivity and evaluate the bioreactor stability in the tested conditions, the flow system was left to operate for 24 h for the preparation of **4d** finally obtaining 2.33 g of pure product (catalyst productivity: 5.33 mmol/mg).

For compounds **4c** and **4f** a very poor conversion was achieved (< 5%), also in flow mode. Lower transformation of serotonin in comparison with tryptamine and 5'-methoxy tryptamine was already noticed using less hindered acyl donors (*i.e.,* EtOAc, VinylAc) and MsAcT as catalyst.[14]

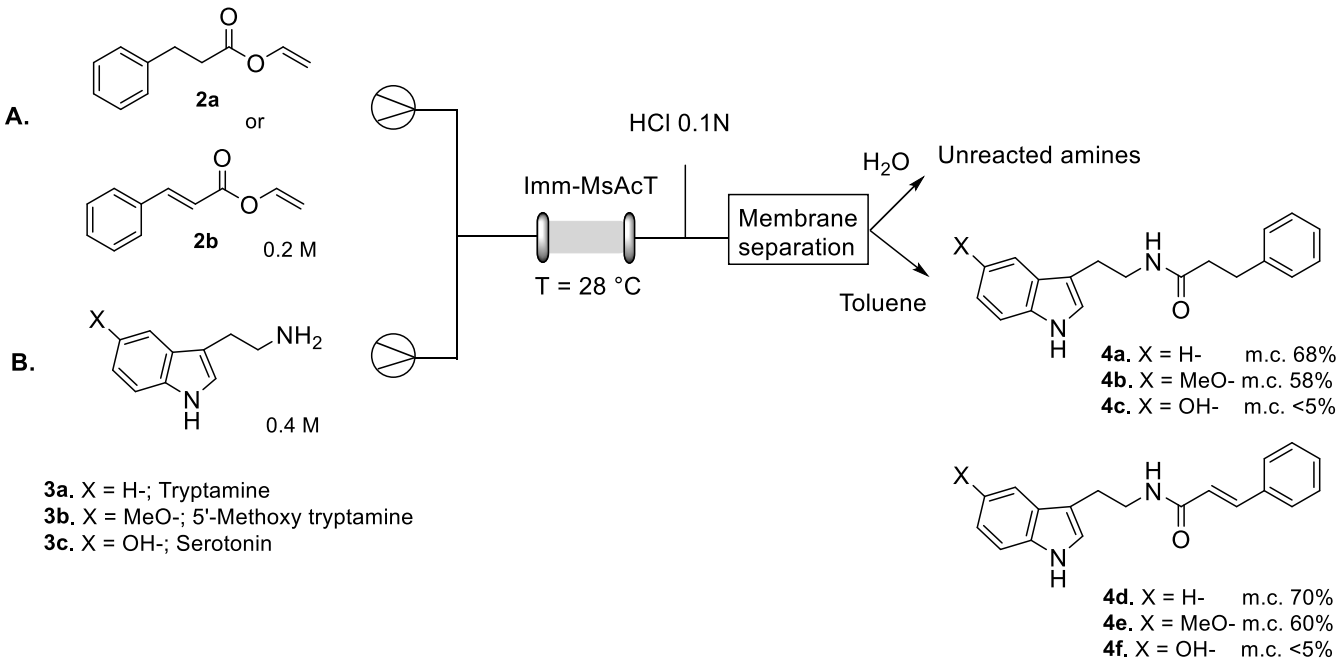


Figure 2. Preparation of cinnamoyl tryptamines. **A.** Solution of vinyl esters **2a** or **2b** (0.2 M in toluene). **B.** Solution of tryptamines **3a**, **3b** or **3c** (0.4 M in toluene).

The clear preference for tryptamine independently from the acyl donor provided, allowed us to perform computational analysis using the simplest possible system: MsAct complexed with the intermediate acetylated serine and each of the substrates. According to the docking studies, tryptamine has the most favourable binding mode in the active site, with the lowest docking score (-8.3 kcal/mol), followed by 5'-methoxy tryptamine (-7.4 Kcal/mol) and serotonin (-7.2 kcal/mol). Although the score suggests a similar fit of serotonin and 5'-methoxy tryptamine, a closer investigation shows that the substrates have very different orientations. While the serotonin

hydroxyl group sits on the back pocket of the active site, formed by hydrophilic residues (*i.e.,* Asp62, Thr64, Asn94 and Lys97), the methoxy group of 5'-methoxy tryptamine is directed towards the front, where more hydrophobic residues are present (*i.e.,* Trp149, Phe174 from one chain; Leu109 and Ser112 from the adjacent chain). Moreover, a further stabilization of 5'-methoxy tryptamine is possible through π-π stacking interactions with Phe174.

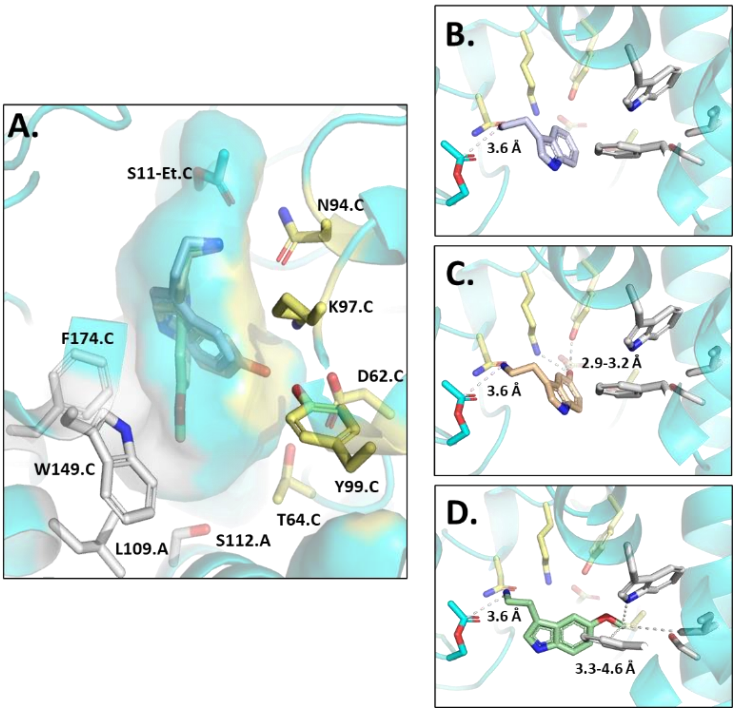
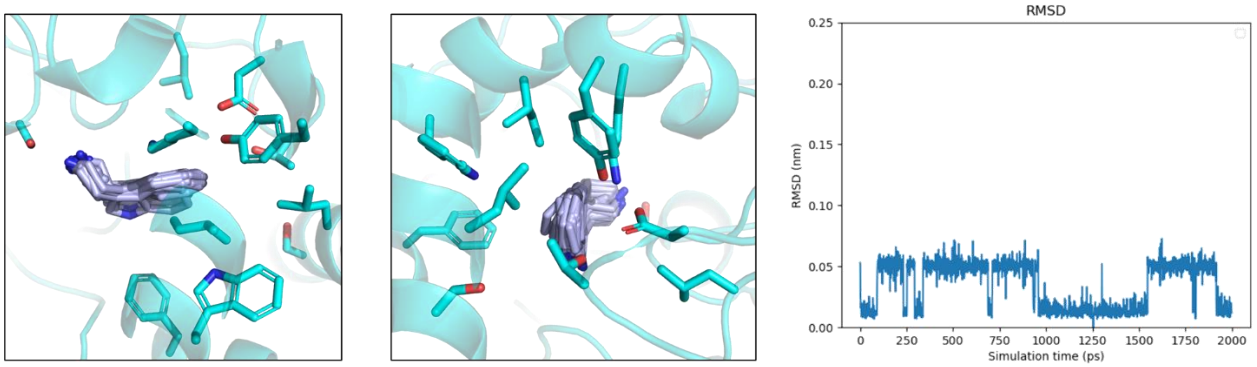


Figure 3. A. Sketch of the catalytic pocket of MsAcT after the first half reaction, where the acetyl group has been transferred to the catalytic serine together with the docked tryptamine (light blue), serotonin (teal) and 5'-methoxy tryptamine (light green). The side chain of the residues forming the hydrophilic (yellow) and the hydrophobic (gray) part of the catalytic pocket are shown. **B.** Details of the docking of tryptamine. **C.** Detail of the docking of serotonin. The distance to the residues responsible for the stabilization of the alcohol group are shown. **D.** Details of the docking of 5'-methoxy tryptamine. The residues interacting with the methoxy group are shown together with the distance.

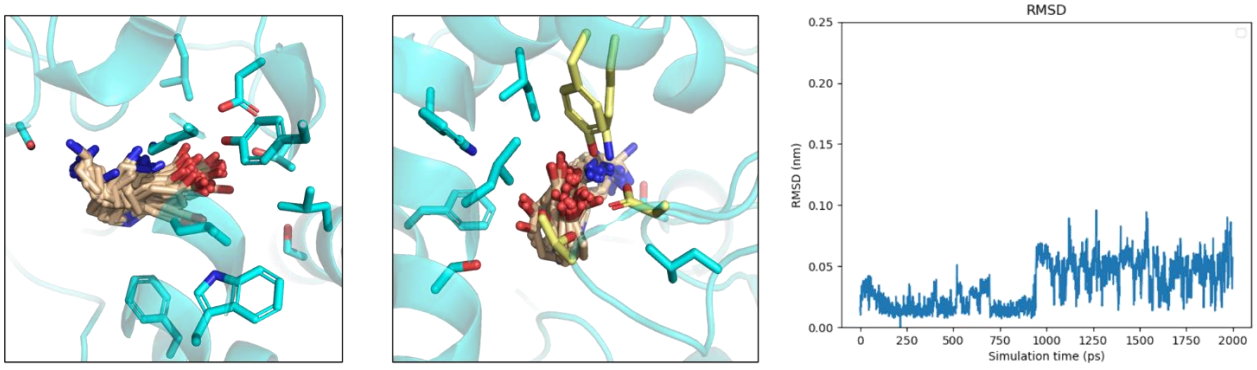
While docking is useful to have a snapshot of the possible catalytic conformation and binding of the substrate, it does not consider the mobility of the protein residues. To gain further insight in the substrate stabilization, molecular dynamic (MD) simulations were performed for 2 ns starting from the docked substrates in the active site and using the Enlighten2 tool.[26] Confirming the initial observations, the three substrates present different behaviours (Fig. 4). For the whole duration of the simulations, tryptamine maintained the same position corresponding to a potentially active catalytic conformation. On the other hand, both serotonin and 5'-methoxy tryptamine showed

variations in their positioning. While both molecules presented similar mobility on the terminal amino group, which could relate to a slower conversion when compared to tryptamine, the mobility of the 5'-substituent was indeed different in the two molecules. As hinted in the docking studies, the presence of a methoxy group translated into a lower variability in the substrate positioning. Interactions with both Phe174 and Trp149, significantly stabilize the indole ring and the hydrophobic methoxy group, while the alcohol seems to establish just transient and continuously shifting hydrogen bonds firstly with Thr62 and subsequently with the Trp107 and Lys95 side chains. These interchangeable interactions allow for a higher mobility of the serotonin in the active site thus explaining its lower reactivity.

A.



B.



C.

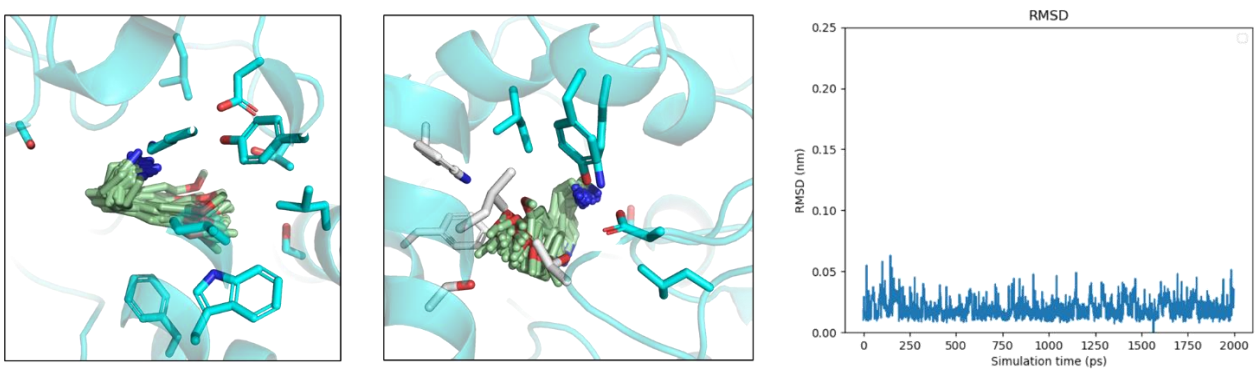


Figure 4. Details of the position of the three substrates: tryptamine (**A**), serotonin (**B**) and 5'-methoxy tryptamine (**C**) during 2 ns simulations. The state every 50 ps is shown and the root mean square deviation (RMSD) of the substrate against its centroid during the simulation is plotted.

Conclusions

An efficient flow-based chemo-enzymatic synthesis has been developed for the preparation of cinnamoyl tryptamines, which are emerging for their cosmetic potential as hyperpigmentation-correcting ingredients. In line with the increasing emphasis placed today on biocatalyst mediated reactions, this system exploits imm-MsAcT, an immobilized acyl transferase from *Mycobacterium smegmatis,* as a key versatile catalyst. Due to the scarce solubility of the starting material, pure toluene was employed demonstrating the enzyme high stability and reusability. The acyl donors, so far prepared just in batch, have been produced in flow mode through Pd(II)-catalyzed reactions reducing procedure time and the cost. In-line work-up was added downstream the process making it fast, safe and easy to handle, while enhancing its automation. Using a small column reactor (1.2 mL), the desired amides were obtained in gram scale (24 h, 115 mL collected), while multi-gram preparation can be achieved by simply increasing the reactor size. The combination of flow facilities and closed-loop strategies allows to operate with a minimum amount of solvents and to recover the unreacted substrates, dramatically impacting on the process sustainability. In addition, computational studies and MD simulations gave a better understanding of the MsAcT catalytic site and substrate reactivity, highlighting the reasons behind the enzymatic preference for tryptamine. The difference between serotonin and 5'-methoxy tryptamine due to their stabilization in the active site was also shown. The availability of robust synthetic methodologies, allowing for a fast preparation (15 min) of cinnamoyl tryptamines in large quantity (0.1 M) would validate their benefits in cosmetic formulations as well as their potential biological properties (*e.g.,* UV protecting, antioxidant, antimicrobial, and anti-inflammatory).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Experimental

1. *Materials and Methods*

All reagents and solvents were purchased from commercial suppliers and used without any further purification. The R-series modular flow chemistry system (Vapourtec®) equipped with Omnifit® glass columns (6.6 mm i.d × 100 mm length). NMR spectra were recorded on a Bruker Avance AV-II 500 MHz or Bruker AV III HD 500 MHz spectrometers using the residual signal of the deuterated solvent as internal standard. ^{1}H chemical shifts (δ) are expressed in ppm, and coupling constants (J) in hertz (Hz). Merck Silica gel 60 F254 plates were used for analytical TLC; flash column chromatography was performed on Merck Silica gel (200–400 mesh). Amberlyst® A 21 resin (anion exchange resin, matrix: styrene divinylbenzene, functional group: dimethylbenzylamine) and Pd EnCat® 40 (microencaplulated $Pd(OAc)_2$ within polyurea matrix) were purchased from Sigma Aldrich. Agarose (6BCL) was purchased from Agarose Bead Technologies (ABT), Madrid, Spain.

Enzyme preparation and immobilization

MsAcT was expressed and purified as previously reported.[23] The enzyme was immobilized and its activity assayed as described by Contente et al.[14]

2. *Small-scale batch bioreactions*

Batch reactions using imm-MsAcT were performed in 10 mL screw cap tubes; 1 mL reaction mixture in toluene, containing 0.2 M amine, 100 mg (1 mg/$g_{agarose}$) of imm-enzyme, 0.1 M of acyl donor were left under gentle agitation at 28 °C. 50 µL aliquots were collected at different reaction times (30 min, 1 h, 2 h, 4 h, 6 h, 24 h) and monitored by TLC (CH_2Cl_2/MeOH 9:1 + 0.1% TEA). After evaporation, the samples were re-suspended in the mobile phase for HPLC analysis (see below).

3. *Flow synthesis of vinyl cinnamates*

An Omnifit® glass column (6.6. i.d.) was packed with commercially available immobilized $Pd(OAc)_2$. A solution of hydroxycinnamic acid or *trans*-cinnamic acid (10 mmol) and vinyl acetate (160 mmol) in 10 mL of THF, and H_2SO_4 20% *w/w* in 10 mL of THF were prepared and degassed. The two solutions were mixed in a T-tube and the resulting flow stream was directed into the first PBR (packed bed reactor volume: 1.5 mL). After 30 min of residence time, the exiting flow stream entered the second PBR filled with Amberlist® A 21 resin in order to trap the unreacted and newly formed acids (packed bed reactor volume: 1.5 mL). Through a second inlet of NaOH 1N (flow rate: 0.1 mL/min), cinnamic acids were recovered as salt form. The final vinyl esters **2a** and **2b** were obtained after column chromatography (*n*-hexane: EtOAc 9:1) as a clear liquid (70% isolated yield) and a pale-yellow liquid (67% isolated yield), respectively.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.75 (m, 2H), 3.02 (t, J= 7.5 Hz, 2H), 4.65 (dd, J= 6.4, 1.9 Hz, 1H), 5.02 (dd, J= 14.0, 1.9 Hz, 1H), 7.23-7.35 (m, 6H) ppm. Proton NMR data is in agreement with literature.[27]

^{1}H-NMR (500 MHz, $CDCl_3$): δ 4.65 (dd, J= 6.4, 1.9 Hz, 1H), 5.02 (dd, J= 14.0, 1.9 Hz, 1H), 6.58 (d, J= 16.0 Hz, 1H), 7.39- 7.49 (m, 4H), 7.50-7.62 (d, J= 8.6 Hz, 2H), 7.85 (d, J= 16.0 Hz, 1H) ppm. Proton NMR data is in agreement with literature.[28]

4. *Flow synthesis of cinnamoyl tryptamines*

An Omnifit® glass column (6.6 mm i.d.) was filled with 1.5 g of imm-MsAcT (1 mg/g). A 0.4 M amine solution and 0.2 M of acyl donor both in toluene were prepared. The two solutions were

mixed in a T-piece and the resulting segmented flow stream directed into the column packed with the biocatalyst (packed bed reactor volume: 1.2 mL). The flow rate was varied and optimized. An in-line extraction was performed using a Zaiput liquid/liquid separator and an inlet of HCl 1N (flow rate: 0.19 mL min^{-1}) was mixed to the exiting reaction flow stream using a T-junction. Both the organic and aqueous phase were analyzed by HPLC using the below reported conditions. The organic phase, containing the amide, was evaporated under reduced pressure, and purified through column chromatography (*n*-hexane:EtOAc 8:2 -> 1/1) yielding the desired products **4a**: 68%; **4b**: 58%; **4d**: 70%; **4e**: 60%.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.40 (t, *J*= 7.5 Hz, 2H), 2.89-3.01 (m, 4H), 3.61 (dt, *J*= 6.5, 6.5 Hz, 2H), 5.60 (br, 1H), 6.82 (s, 1H), 7.02-7.32 (m, 7H), 7.37 (d, *J*= 8.1 Hz, 1H), 7.58 (d, *J*= 7.9 Hz, 1H), 8.51 (br, 1H) ppm. Proton NMR data is in agreement with literature.[29]

^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.41 (t, *J*= 7.6 Hz, 2H), 2.90-3.10 (m, 4H), 3.62 (dt, *J*= 6.5, 6.5 Hz, 2H), 3.83 (s, 3H), 5.60 (br, 1H), 6.80 (s, 1H), 7.02-7.33 (m, 6H), 7.38 (d, *J*= 8.0 Hz, 1H), 7.59 (d, *J*= 7.8 Hz, 1H), 8.52 (br, 1H) ppm. Proton NMR data is in agreement with literature.[29]

^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.01 (t, *J*= 6.7 Hz, 2H), 3.71 (q, *J*= 6.7 Hz, 2H), 5.89 (br, 1H), 6.28 (d, *J*= 15.0, 1H), 7.02 (d, *J*= 1.6 Hz, 1H), 7.10-7.16 (m, 1H), 7.17-7.26 (m, 1H), 7.32-7.46 (m, 6H), 7.57-7.62 (m, 2H) 8.37 (br, 1H) ppm. Proton NMR data is in agreement with literature.[30]

^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.02 (t, *J*= 6.7 Hz, 2H), 3.70 (q, *J*= 6.7 Hz, 2H), 3.83 (s, 3H), 5.88 (br, 1H), 6.29 (d, *J*= 15.2, 1H), 7.02 (d, *J*= 1.6 Hz, 1H), 7.10-7.15 (m, 1H), 7.17-7.25 (m, 1H), 7.31-7.45 (m, 5H), 7.55-7.61 (m, 2H), 8.36 (br, 1H) ppm. Proton NMR data is in agreement with literature.[30]

5. HPLC analysis

HPLC Analysis were performed using a Merck-Hitachi 655 A-12 Liquid Cromatograph with L-5000 LC controller and 655A Variable Wavelength UV monitor detector. Column: Chiralpack IC (250 x 4.6 mm x 3 µm). Eluent: *n*-hexane/EtOH 70:30 + 0.1% TEA, λ = 250 or 280 nm, flow rate: 1 mL/min). The retention times were: tryptamine (**3a**) 7.7 min, cinnamoyl tryptamine (**4a**) 5.2 min, *trans*-cinnamoyl tryptamine (**4d**) 5.0 min; 5'-methoxy tryptamine (**3b**) 9.1 min, cinnamoyl 5'-methoxytryptamine (**4b**) 6.4, *trans*-cinnamoyl 5-methoxytryptamine (**4e**) 6.1 min; serotonin (**3c**) 9.8 min.

6. Computational studies

Docking was performed using Autodock Vina[31] in combination with UCSF Chimera software[32] for the visualization, using MsAcT 3D structure present in the protein data bank (PDB id: 2q0s) and manually building the substrates based on their SMILES notation. To perform the molecular dynamics studies, the Enlighten2 plugin[26] for PyMOL[33] was used. Simulations were performed with mobility only on the residues at 12Å surrounding the docked substrate. After minimization of the system, the MD simulation was performed for 2ns. Results were analyzed using MDtraj package.[34]

Acknowledgments

The authors would like to thank Prof. Paradisi (DCBP, University of Bern) for the support and the fruitful discussions.

References

1. Gambacorta G, Sharley JS, Baxendale IR. *Beilstein J Org Chem*. 2021; 17: 1181–1312.
2. Baumann M, Moody TS, Smyth M, Wharry S. *Org Process Res Dev*. 2020; 24: 1802–1813.
3. Ley S V., Chen Y, Robinson A, Otter B, Godineau E, Battilocchio C. *Org Process Res Dev*. 2021; 25: 713–720.
4. Sheldon RA, Woodley JM. *Chem Rev*. 2018; 118: 801–838.
5. Sheldon RA, Brady D. *ChemSusChem*. 2019; 12: 2859–2881.
6. Garcia-Verdugo E, Porcar R, Luis S V., Lozano P. In *Flow Chemistry: Integrated Approaches for Practical Applications* 2020; pp 50–85.
7. Contente ML, Paradisi F. *Nat Catal*. 2018; 1: 452–459.
8. Santi M, Sancineto L, Nascimento V, Azeredo JB, Orozco EVM, Andrade LH, Gröger H, Santi C. *Int J Mol Sci*. 2021; 22: 1–32.
9. Znidarsic-Plaz P. *J Flow Chem*. 2018; 7: 111–117.
10. De Vitis V, Dall'Oglio F, Pinto A, De Micheli C, Molinari F, Conti P, Romano D, Tamborini L. *ChemistryOpen*. 2017; 6: 668–673.
11. Itabaiana I, Leal ICR, Miranda LSM, De Souza ROMA. *J Flow Chem*. 2013; 3: 122–126.
12. Contente ML, Tamborini L, Molinari F, Paradisi F. *J Flow Chem*. 2020; 10: 235–240.
13. Annunziata F, Contente ML, Pinna C, Tamborini L, Pinto A. *Antioxidants*. 2021; 10: 1142–1150.
14. Contente ML, Farris S, Tamborini L, Molinari F, Paradisi F. *Green Chem*. 2019; 21: 3263–3266.
15. Annunziata F, Contente ML, Betti D, Pinna C, Molinari F, Tamborini L, Pinto A. *Catalysts*. 2020; 10: 1–8.
16. Taofiq O, González-Paramás AM, Barreiro MF, Ferreira ICFR, McPhee DJ. *Molecules*. 2017; 22: 281–305.
17. Roh JS, Han JY, Kim JH, Hwang JK. *Biol Pharm Bull*. 2004; 27: 1976–1978.
18. Couteau C, Coiffard L. *Cosmetics*. 2016; 3.
19. Chemat F, Vian MA, Cravotto G. *Int J Mol Sci*. 2012; 13: 8615–8627.
20. Lee SJ, Sim GY, Lee Y, Kim BG, Ahn JH. *J Ind Microbiol Biotechnol*. 2017; 44: 1551–1560.
21. Pattabiraman VR, Bode JW. *Nature*. 2011; 480: 471–479.
22. Benitez-Mateos AI, Contente ML. *Catalysts*. 2021; 11: 814–824.
23. Contente ML, Pinto A, Molinari F, Paradisi F. *Adv Synth Catal*. 2018; 360: 4814–4819.
24. de Leeuw N, Torrelo G, Bisterfeld C, Resch V, Mestrom L, Straulino E, van der Weel L, Hanefeld U. *Adv Synth Catal*. 2018; 360: 242–249.
25. Szymańska K, Odrozek K, Zniszczoł A, Torrelo G, Resch V, Hanefeld U, Jarzębski AB. *Catal Sci Technol*. 2016; 6: 4882–4888.
26. Zinovjev K, Van Der Kamp MW. *Bioinformatics*. 2020; 36: 5104–5106.
27. Savile CK, Kazlauskas RJ. *J Am Chem Soc*. 2005; 127: 12228–12229.

28. Kadidae LO, Usami A, Honda M. *Asian J Chem*. 2018; 30: 589–593.
29. Kiviranta PH, Salo HS, Leppänen J, Rinne VM, Kyrylenko S, Kuusisto E, Suuronen T, Salminen A, Poso A, Lahtela-Kakkonen M, Wallén EAA. *Bioorganic Med Chem*. 2008; 16: 8054–8062.
30. De Vita D, Simonetti G, Pandolfi F, Costi R, Di Santo R, D'Auria FD, Scipione L. *Bioorganic Med Chem Lett*. 2016; 26: 5931–5935.
31. Trott O, Olson AJ. *J Comput Chem*. 2009; 31: NA-NA.
32. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. *J Comput Chem*. 2004; 25: 1605–1612.
33. DeLano WL. *CCP4 Newsl protein Crystallogr*. 2002; 40: 82–92.
34. McGibbon RT, Beauchamp KA, Harrigan MP, Klein C, Swails JM, Hernández CX, Schwantes CR, Wang LP, Lane TJ, Pande VS. *Biophys J*. 2015; 109: 1528–1532.