


Experimental and in silico approaches to study CES substrate selectivity

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Abstract

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Keywords: keyword 1; keyword 2; keyword 3 (List three to ten pertinent keywords specific to the article; yet reasonably common within the subject discipline.)

1. Introduction

Human Carboxylesterases (CES, EC 3.1.1.1) are serine hydrolase enzymes responsible for the metabolism and biotransformation of diverse endogenous and xenobiotic substrates containing esters, thioesters, amides, carbonates and carbamates moieties in their structures[1–3]. Based on amino acid sequence homology, human CES are classified into five isoforms (CES1–CES5). Among these, CES1 and CES2 are the most clinically relevant isoforms[4–6].

CES1 and CES2 share 47% of protein sequence identity but exhibit distinct substrate specificities and tissue distributions. Specifically, CES1 is primarily expressed in the liver, whereas CES2 is predominantly found in the intestines[1,3,4,7]. It is widely accepted in the literature that the substrate binding and hydrolysis selectivity of each isoform are primarily determined by the size of the acyl and alkyl moieties of the respective substrate molecular structures[4,6–8]. In this context, CES1 preferentially catalyzes the hydrolysis of substrates with smaller alkyl than acyl groups, as seen with drugs and prodrugs like clopidogrel, oseltamivir and meperidine[2,8,9]. Conversely, CES2 tends to hydrolyze compounds with smaller acyl than alkyl groups, examples of which include halperidol, procaine and flutamide[1,3,8].

Despite this general trend, several exceptions to substrate selectivity have been reported. Notably, drugs and prodrugs like irinotecan, propanil, oxybutinin and procaine have been shown to be metabolized by both CES isoforms with similar efficiency [8,10]. Another noteworthy example is the differential CES-mediated biotransformation rates of

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pyrethroid derivatives, where *cis* and *trans* isomers of permethrin exhibit distinct hydrolysis pattern by CES1 and CES2, despite having identical acyl and alkyl group sizes[11].

Given the critical role of CES-mediated catalysis in various physiological processes, understanding the structural determinants of substrate specificity among CES isoforms is crucial for several areas of research. One such area is the design of prodrugs with optimized biopharmaceutical profiles. This approach aims to avoid undesirable early biotransformation of the prodrug or to enable site-specific bioactivation targeting a particular CES isoform[2,12]. In addition, the development of specific inhibitors for CES1 and CES2 offers promising therapeutic potential for managing metabolic diseases. For instance, the CES1 inhibitor GR148672X has been explored as a candidate for treating hypertriglyceridemia, obesity and atherosclerosis[13].

In the field of medical imaging, there is growing interest in the development of fluorescent biological probes for selective detection of CES1 and CES2 activity[5,14–17]. Over recent years, the selective imaging of *in vivo* enzyme activity has emerged as a powerful method for the study of biological systems, due to its ability to provide real-time, noninvasive monitoring within living organisms[5,14–17]. In this context, the design of fluorescent probes that specifically target CES1 and CES2 offers a promising strategy for visualizing their activity in complex biological systems.

Enzymology has long been a foundational field for studying enzyme structure and function, as well as advancing our understanding of biological phenomena such as intermediary metabolism, molecular biology, and cellular signaling and regulation[18]. Early enzymology focused primarily on experimental techniques aimed at analyzing the catalytic properties and molecular specificity of enzymes[18]. The first one studies the thermodynamic and kinetic of the enzymatic reaction, measuring free-energy of reaction and activation. The second studies the specificity of different molecules for the enzymes, not only limited to the substrates, but also any other molecule that satisfy the specificity criteria for the enzyme, as is the case for potential inhibitors[18].

Due to the complexity of enzymes and the challenges associated with studying biomolecular reactions, many questions and mechanisms remain unclear. Computational enzymology, defined as the study of enzymes and the their reactions mechanisms by molecular modeling and simulation [19], has the unique potential to investigate the dynamic behavior and reactions of biomolecules at atomic resolution. This approach can address unresolved issues by complementing and interpreting findings from experimental enzymology[19–21].

Since the beginning in 1976, with the pioneering work of Warshel and Levitt (Nobel laureates in 2013), computational enzymology has rapidly evolved over the past two decades. This progress has been driven by close collaboration between experimental and computational enzymologists, enhancing our ability to explain and interpret experimental data[19–21].

Building on the symbiotic relationship between experimental and computational enzymology, this review will focus on the recent advancements in methodologies developed to study substrate selectivity between the two most important isoforms of human carboxylesterase: CES1 and CES2.

2. Part I: Experimental enzymology

2.1. Kinetic parameters

During the study of the enzymatic properties of different substrates, there are important kinetic parameters determine by the Michaelis-Menten fundamental equation of enzyme kinetics[18,22]. The first parameter is the **dissociation constant**, also known as Michaelis constant (K_M), which reflects the affinity between the enzyme and the correspond-

ing substrate. A lower the K_M value indicates stronger binding within the enzyme-substrate complex [2,18,23,24]. The second key kinetic parameter is the **turnover number**, also referred as catalytic constant (k_{cat}). This value represents the number of catalytic cycles the enzyme can complete per unit of time when it is fully saturated with substrate. A higher k_{cat} value signifies greater substrate turnover and more efficient metabolism by the enzyme [2,18,23,24].

The ratio between these two kinetic parameters (k_{cat} / K_M) is known as **specificity constant**, as it reflects the ability of an enzyme to discriminate between structurally similar substrates. A higher k_{cat} / K_M ratio indicates that the enzyme shows high affinity (low K_M value) and high catalytic rate for the substrate. Because of this, the specificity constant is often used as a measure of **catalytic efficiency**, indicating if the ligand is a good or poor substrate for the enzyme[18,24].

To obtain these kinetic parameters to quantify the substrate selectivity for a specific enzyme, experimental enzymology is used to measured the progress of an enzyme-catalyzed reaction. Like any other chemical reaction, the progress can be monitored either by measuring the formation of the product or the consumption of the substrate. Reliable detection methods for product formation or substrate depletion are essential for a successful enzyme assay[18].

2.2. Enzyme sources

This section outlines the different CES enzymatic sources used in various analytical methodologies for calculating kinetic parameters. The two primary sources are *ex vivo* tissues, including purified human tissues and human microsomes, and pure recombinant enzymes (Table 1).

Table 1. Enzymatic sources and analytical methods used for the exploration of CES kinetic parameters on different substrates.

Substrates	Ex vivo tissue		Recombinant enzyme	Analytical method	Reference
	Human tissue	Human microsomes			
Irinotecan	Liver	-	-	HPLC-FL	[25,26]
Methylphenidate	Liver	-	CES1/CES2	LC/MS	[27]
Oseltamivir	-	HLM/HIM	-	HPLC-UV	[8,28]
Temocapril	-	HLM/HIM	CES1/CES2	HPLC-UV	[8,29]
Aspirin	-	HLM/HIM	CES1/CES2	HPLC-UV	[29,30]
Clopidogrel	-	HLM/HIM	CES1/CES2	HPLC-UV	[8,30]
Flutamide	-	HLM/HIM	CES1/CES2	HPLC-UV	[31,32]
Pyrethroids	-	-	CES1/CES2	FL	[23,33]
Fluorescein diacetate	-	HLM/HIM	CES1/CES2	FL	[34]
Plasugrel	-	-	CES1/CES2	LC/MS	[35]
Heroin	-	-	CES1/CES2	HPLC-UV	[36]
Cocaine	-	-	CES1/CES2	HPLC-UV	[36,37]
Oxybutynin	-	HLM	CES1/CES2	LC/MS	[38]
Prilocaine	-	HLM	CES1/CES2	HPLC-UV	[39]
Lidocaine	-	HLM	CES1/CES2	HPLC-UV	[39]
Clofibrate	-	-	CES1/CES2	HPLC-UV	[8]
Fenofibrate	-	HLM/HIM	CES1/CES2	HPLC-UV	[8,40]
Imidapril	-	-	CES1/CES2	HPLC-UV	[8]
Enalapril	-	HLM	CES1/CES2	LC/MS	[41]
Sacubitril	Liver	-	CES1/CES2	LC/MS	[42]
Anordrin	-	HLM/HIM	CES1/CES2	LC/MS	[43]

HLM: Human liver microsomes. HIM: human intestine microsomes. FL: Fluorescence

2.2.1. *Ex vivo* tissue

Since the early 2000's, the enzymatic studies of CES1 and CES2 substrate hydrolysis have relied on purified isoforms sourced from human liver. In several studies, human liver tissue is processed by homogenization and centrifugation, followed by separation of CES1 and CES2 isoforms using various chromatographic columns[25–27]. Using this method, it was determined that the produg irinotecan, along with other metabolites, is primarily bioactivated by human CES2 isoform [25,26].

The second source of CES is human tissue microsomes, which are small vesicles derived from fragmented cell membranes, mainly endoplasmic reticulum. These human microsomes can be obtained by differential centrifugation of the corresponding tissue or purchased from different biological supply companies. Human liver microsomes (HLM) are commonly used as enzyme source for measuring metabolic stability, as they contain key metabolizing enzymes, including CES. As mentioned in the introduction, CES1 is predominantly expressed in the liver, making HLM a valuable source for studies of CES1 substrate selectivity. Following similar tissue distribution patterns, human intestines microsomes (HIM) have been used as a source for CES2 in selectivity assays. Using this approach, studies have shown that the antiviral produgs oseltamivir and temocapril are preferentially activated by CES1 (HLM) over CES2 (HIM)[28,29].

2.2.2. Recombinant enzyme

Shortly after the use of human tissue as enzyme source, it became evident that a more purified version of both CES isoforms were necessary to performed accurate selectivity enzymatic experiments with different substrates. Morton and Potter developed a method for cloning and expressing CES using baculovirus to infect *Spodoptera frugiperda* insect cells [44]. This technique enabled the production of recombinant CES1 and CES2, which were then used to measure the enzymatic properties of several substrates (Table1). The hydrolysis of pyrethroids by human CES1 and CES2 have been studied using this recombinant enzyme source [23,33]. Additionally, the hydrolysis specificity of CES1 and CES2 for drugs of abuse, such as heroin and cocaine[36], as well as other drugs and prodrugs, have also been studied using recombinant enzymes (Table1)[8,38,39].

As recombinant CES enzymes became commercially available from multiple suppliers, research groups were able to continue exploring the metabolism selectivity of CES isoforms across different substrates. For example, studies on the angiotensin-converting enzyme inhibitors enalapril and ramipril showed that both drugs were selectively hydrolyzed by CES1[41]. Recombinant CES enzymes have also been used to determine the predominant role of CES isoforms in the human bioactivation of prodrugs such as sacubitril[42] and anordrin[43]. Additionally, recombinant CES enzyme have facilitated pre-clinical evaluations of the bioactivation rates for structurally designed prodrugs, including atorvastatin[45,46], indomethacin[47,48] and haloperidol[49].

2.3. Reported analytical methods

As mentioned earlier, obtaining accurate kinetic parameters requires a reliable analytical method to quantify the progress of the enzyme-catalyzed reaction. In this section, the different analytical methods used for the determination of the described kinetic parameters for different substrates and both CES isoforms will be described.

2.3.1. Spectrophotometry

Spectrophotometric methods have been widely used to quantitatively determine total CES activity, by measuring the absorbance of *p*-nitrophenol, produced through the hydrolysis of *p*-nitrophenyl acetate (pNPA), at 405 nm (Figure 1)[36,50,51]. This approach

was applied to the functional characterization of recombinant human CES expressed in *E. coli* as an alternative method for obtaining the human enzyme[51].

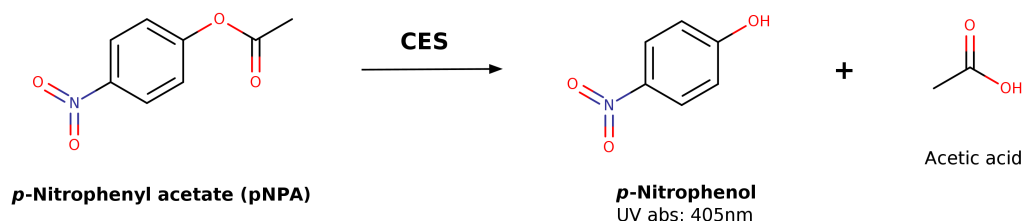


Figure 1. Hydrolysis of *p*-nitrophenyl acetate (pNPA) by CES to produced *p*-nitrophenol.

The absorbance properties of *p*-nitrophenol have also been used to evaluate the kinetic parameters of various *p*-nitrophenyl esters derivatives with CES1 and CES2[36]. The authors observed a correlation between the affinity constant (K_M) and the calculated water/octanol partition coefficients (clogP) values, concluding that the affinity of the substrates for both CES isoforms is directly related to their lipophilicity properties[36]. In addition, kinetic data for naphthyl esters derivatives were obtained by measuring the formation of naphthol at 230nm [50].

This method is simple and rapid, but it has several disadvantages. One key issue is the potential overlapping interference between substrates and products, which complicates experiments in complex biological systems. Additionally, the method requires a larger amount of enzyme, as it is performed in a UV cuvette with a total volume of 1 ml[51].

2.3.2. Fluorescence

In contrast to the previously described analytical method, the fluorescent probe-based approach is not only simple but also highly selective and sensitive. These probes quantify CES activity by detecting changes in fluorescence intensity. Known as "off-on" fluorescent probes, they initially exhibited little or no fluorescence, but the hydrolyzed products release strong fluorescence in presence of CES.

This methodology has been used to determine the kinetic parameters of pyrethroids-like substrates containing 6-methoxy-2-naphthaldehyde. The fluorescence of this moiety is measured with an excitation wavelength of 330 nm and an emission wavelength of 465 nm[23]. In this study, it was observed that the stereoisomeric centers of these derivatives presented a differential impact on hydrolysis by the two CES isoforms. Specifically, the presence of an (*R*)-enantiomer carbon adjacent to the ester carbonyl carbon resulted in a greater preference for CES2 hydrolysis than CES1[23]. These findings highlight the importance of the three-dimensional disposition of the substrate groups within the catalytic site of the enzyme for CES hydrolysis selectivity.

In another study, fluorescein diacetate was used as a substrate to assess CES1 and CES2 selectivity. The hydrolysis of fluorescein diacetate by the CES enzymes releases fluorescein, which was quantify at an excitation wavelength of 483 nm and emission at 525 nm. The results indicated that fluorescein diacetate is a selective CES2 substrate (Figure 2)[34].

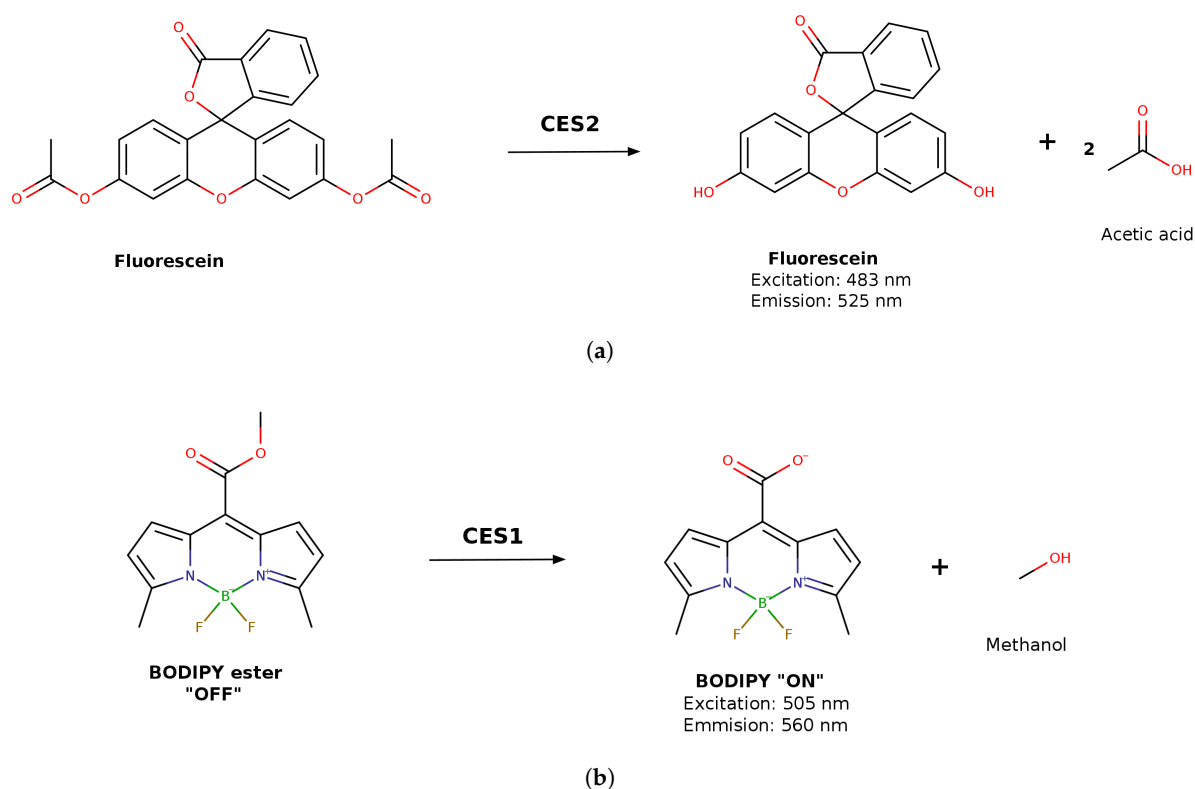


Figure 2. hydrolysis of fluorescent probe substrates. (a) Fluorescein diacetate. (b) BODIPY ester.

The high fluorescence quantum yield and photochemical stability of BODIPY dyes make them excellent candidates for this analytical methodology. As a result, a BODIPY ester was designed as a specific substrate for CES1. The acid product formed after CES hydrolysis was used to measure the kinetic parameters at an excitation wavelength of 505 nm and emission at 560 nm (Figure 2)[52].

2.3.3. Chromatography

Diverse chromatographic methods have been widely used to determine the kinetic parameters of numerous substrates (e.g., drugs, prodrugs, pyrethroids) due to their high sensitivity, accuracy, rapid analysis and reproducibility. One additional advantage is the ability to separate substrate from products for precise quantification. The two most commonly used techniques are the high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC/MS).

In the case of HPLC, an additional detection technique is required to quantify the different ligands after chromatographic separation, with the two previously described methodologies being the most frequently used. The spectrophotometric methodology usually implements an UV-detector (HPLC-UV), as most ligands exhibit absorbance in the ultraviolet spectrum. This methodology has been widely used to study the CES kinetic parameters of various therapeutic drugs and prodrugs.

To investigate substrate specificity among CES isoforms, a study was conducted on 13 compounds, including clopidogrel, clofibrate, oseltamivir, mycophenolate mofetil, procaine and temocapril, among others (Table 1), using HPLC with specific conditions for each metabolite (e.g., mobile phase, column and UV wavelength)[8]. This research group also applied the HPLC-UV method to analyze the kinetics of other drugs, such as flutamide[31,32], prilocaine and lidocaine[39]. Based on the structural characteristics of these compounds, the authors proposed the already discussed general substrate selectivity pattern for CES: CES1 preferentially hydrolyzes ligands with smaller alkyl than acyl moiety

(e.g., clofibrate, lidocaine, temocapril), while CES2 favors those with larger alkyl than acyl groups (e.g., flutamide, procaine).

The metabolism of the abuse drugs cocaine and heroine by CES1 and CES2 was also studied using this methodology[36]. For cocaine, hydrolysis was monitored by quantifying the formation of benzoylecgonine and benzoic acid at 235nm. After incubation with both CES isoforms, the results showed that cocaine was exclusively metabolized by CES2, producing benzoic acid and ecgonine methyl ester. The second potential metabolic pathway, forming benzoylecgonine and methanol, was not detected[36].

For heroine, hydrolysis was assess by monitoring the formation of 6-acetylmorphine, also at 235nm. Both CES isoforms were found to hydrolyzed heroin, with CES1 exhibiting a higher catalytic efficiency (k_{cat}/K_M) compared to CES2[36]. This result represents an exception to the general CES1 substrate specificity, as heroin has a larger size alkyl group than acyl moiety in its structure.

A different approach was presented in several studies by Takahashi et al., in which all the investigated substrates were structurally diverse indomethacin-derived prodrugs. The formation of indomethacin, monitored at 254 nm, was used as the analytical marker to determine the kinetic parameters[47,48,53]. By synthesizing prodrugs bearing a variety of alkyl moieties, the authors were able to investigate how different structural features influence the hydrolysis behavior of both CES isoforms. Specifically, they examined: 1) the effect of the steric hindrance on the carbon adjacent to the carbonyl group, 2) the influence of electron density around the carbonyl group and 3) the chiral recognition ability between CES1 and CES2.

Overall, the results indicated that the indomethacin prodrugs were mainly hydrolyzed by CES1, because the drug structure represents the acyl group, which in all the cases was larger than the corresponding alkyl group. However, prodrugs with aryl-containing alkyl moieties exhibited reduced or even no selectivity between CES isoforms, demonstrating that steric hindrance near the ester carbonyl carbon plays a crucial role in determining metabolic selectivity.

This research group also conducted similar studies by synthesizing prodrugs derivatives of haloperidol[49] and atorvastatin [46], using the absorbance properties of these drugs as detection methods for the HPLC technique.

On the other hand, LC/MS does not required an additional method for the quantification of the desired kinetic parameters, as both substrate and product are detected directly by the mass spectrometer following their liquid chromatographic separation. This analytical technique has primarily been used to investigate the metabolic pathway of a diverse set of prodrugs.

Interesting results were observed in the hydrolysis study of capecitabine, a carbamate prodrug of 5-fluoruracil used in the treatment of colorectal cancer[54]. The study showed that capecitabine is hydrolyzed equally by both CES1 and CES2, suggesting that substrates containing carbamate groups can be metabolized by either isoform, independent of the size of their acyl or alkyl moieties. Other prodrugs studied include plasugrel, which exhibited selectivity for CES2[35], sacubitril, selectively activated by CES1[42], and anordrin, which showed similar hydrolysis parameters with both CES isoforms[43].

In a more recent study, the hydrolysis characteristics of cocaine by CES1 were re-examined using LC/MS methodology[37]. This study revealed that cocaine is hydrolyzed by CES1, producing benzoylecgonine and methanol, which contrast with earlier findings using HPLC-UV, where this hydrolysis product was not detected (Figure 3)[36]. These results suggest that LC/MS provides a more accurate analytical approach for studying cocaine hydrolysis compared to HPLC-UV.

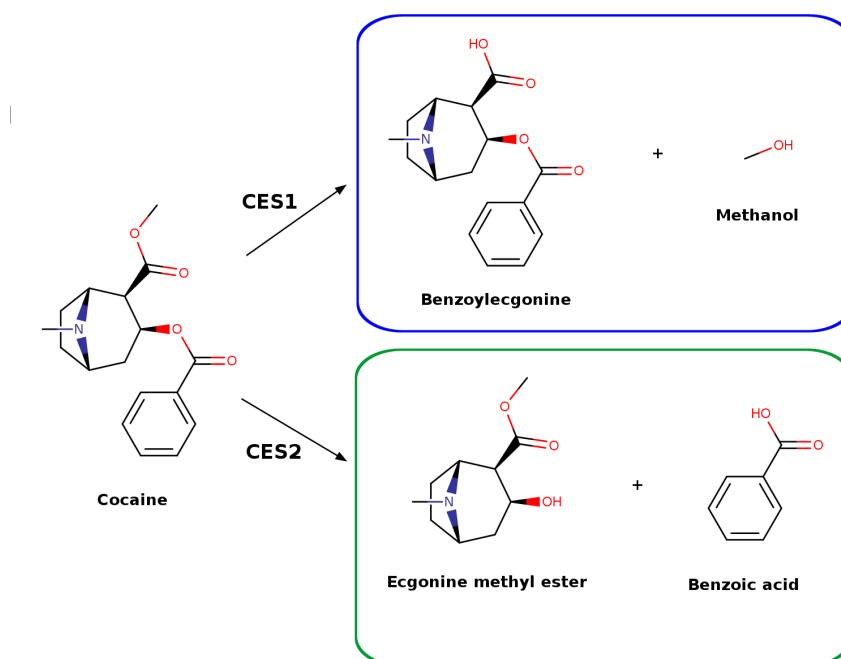


Figure 3. Different obtained cocaine metabolic pathway from CES1 and CES2 hydrolysis experimental studies with HPLC-UV and LC/MS analytical methodologies.

This section highlights the importance of experimental enzymology in studying CES substrate selectivity. The gathered information shows that, despite the general structural rules established for substrate selectivity between CES1 and CES2, several exceptions and underlying structural properties remain unclear. Given that CES binding and hydrolysis involve subtle intermolecular interactions, computational enzymology emerges as a crucial tool for analyzing CES substrate selectivity at the atomistic level.

2.4. Experimental CES kinetic parameter databases

In the era of bioinformatics, providing reliable kinetic parameter data and effective data management systems is essential to support researchers in retrieving enzymatic information from the vast amount of biological data published annually, such as those related to CES enzymes. In this context, online databases have become invaluable tools for granting researchers access to this information. This section will focus on two of the most well-known and widely used online databases for obtaining human CES enzymatic parameters for different substrates.

2.4.1. BRENDA database

The BRAunschweig ENzyme DATABASE (BRENDA) is the oldest collection of enzyme-related data from the scientific literature, established in 1987 at the German National Research Centre for Biotechnology in Braunschweig [55]. Today, the BRENDA website (www.brenda-enzymes.org/) is accessed by over 100,000 users each month. The site is very intuitive, allowing users to search by entering a text, such as enzyme name, ligand name, EC class, inhibitors, etc., or through structured-based queries by drawing substrate/products or ligand substructures[55].

The search for CES (EC 3.1.1.1) enzymatic information on BRENDA, yielded 2079 substrates/products and 67 natural products from the database. From these ligands, 849 K_M values, 550 turnover numbers (k_{cat}) and 232 k_{cat}/K_M values (catalytic efficiency) can be retrieved from diverse bibliographic sources. In addition, the reaction diagrams and references associated with these substrates were provided.

However, two main disadvantage were noted: 1) it is not possible to filtrate information based on the organism origin of the enzyme, and 2) the kinetic information can not easily be separate by CES isoforms. Despite this limitations, BRENDA remains a valuable starting point for searchif kinetic parameters related to CES1 and CES2 substrate hydrolysis.

2.4.2. SABIO-RK database

SABIO-RK is a manually curated database containing enzymatic biochemical reactions and their kinetic parameters (sabiork.h-its.org/). The authors have established that the database is a valuable resource for both experimental and computational enzymology researchers[56]. Data in SABIO-RK are primarily extracted manually from the literature and stored in a structured and standardized format. The database includes essential data to describe the characteristics of biochemical reactions, the corresponding biological source, kinetic properties and experimental conditions[56].

The regular search for CES name in SABIO-RK website returned 519 entries, which is a smaller amount of information compared to BRENDA. The SABIO-RK website offers an advance search feature that allows filtering by various conditions, such as "organisms: *Homo sapiens*", which narrows the results to 168 entries related to human CES data. Additionally, it is possible to filter the results to include only data from recombinant CES enzyme (82 entries). However, like BRENDA database, SABIO-RK also has the limitation of not separating kinetic parameters by CES isoforms. In conclusion, while SABIO-RK contains less information than BRENDA, it offers better organization and more intuitive filtering options, making it easier to retrieve relevant data.

3. Part II: Computational enzymology

3.1. Enzyme sources: structures

At the beginning of any computational molecular modeling substrate-enzyme complex study, the information regarding the structure of both, the enzyme and the substrate, is needed. The structures of the ligands are relatively easy retrieved from different biological databases, such as those mentioned in the previous section. On the other hand, obtaining the structures of the macromolecules (enzymes) can be more challenging. This is because enzyme structures are often distributed across different sources and databases. In this section, the reported sources for obtaining the three-dimensional structures of CES1 and CES2 enzymes will be displayed.

3.1.1. CES1 crystal structures

Since 2003, different crystallographic structures of CES1 have been reported, as summarized in Table 2. The first published structures involved CES1 complexes with ligands from several categories, including metabolites of abuse drugs like cocaine and heroin (homatropine and naloxone methiodide, respectively)[57], therapeutic drugs (tacrine, tamoxifen and mevastatin)[58,59], and endogenous substrates (cholate, taurocholate and coenzyme A) [60] (Table 2). Notably, the crystal structure of CES1-tamoxifen complex revealed that tacrine interacts in the catalytic binding site of CES1 in four different binding modes[58]. These findings highlights the promiscuous nature of CES1, suggesting that its ability to hydrolyzed a variety of substrates is driven by its capacity to interact with these ligands in multiple conformations[58].

Table 2. Information related to CES1 reported crystallographic structures.

Substrates	Classification	Resolution (Å)	PDB code	Reference
Homatropine	M.L.	2.80	1MX5	[57]
Naloxone methiodide	M.L.	2.90	1MX9	[57]
Tacrine	Drug	2.40	1MX1	[58]
Tamoxifen	Drug	3.20	1YA4	[59]
Mevastatin	Drug	3.00	1YA8	[59]
Ethylacetate	M.L.	3.00	1YAH	[59]
Benzil	M.L.	3.20	1YAJ	[59]
Cholate/Palmitate	E.S.	3.00	2DQY	[60]
CoenzymeA	E.S.	2.00	2H7C	[60]
CoenzymeA/Palmitate	E.S.	2.80	2DQZ	[60]
Taurocholate	E.S.	3.20	2DR0	[60]
Soman	N.A.	2.70	2HRQ	[61]
Tabun	N.A.	2.70	2HRR	[61]
Cyclosarin	N.A.	3.10	3K9B	[62]
-	-	2.20	4AB1	[63]
-	-	1.86	5A7F	[64]
-	-	2.67	8EOR	[65]
F-3	C.I.	1.83	9KWL	[66]
F-4	C.I.	1.89	9KWM	[66]

M.L.: Metabolite ligand. E.S.: Endogenous substrate. N.A.: Nerve agent. C.I.: covalent inhibitor.

Different types of covalent ligands have also been studied through crystallographic structures. The first group to be investigated included the organophosphorus nerve agents soman, tabun and cyclosarin [61,62]. More recently, attention shifted to a second family focused on the covalent binding mechanism of serine-selective electrophilic warheads. Two crystal structure were generated showing CES1 covalently bound to 2,2,2-trifluoroacetophenone derivatives at the catalytic serine (Table 2)[66]. Additionally, there are crystallographic structures of CES1 in the absence of substrates[63–65]. One of these structures exhibited the highest resolution of any reported CES1 crystal structure to date (1.86 Å).

For conducting molecular modeling studies focused on substrate selectivity between CES isoforms, the optimal approach is to use the CES1 structure with the highest possible resolution and complexed with non-covalent ligands or in a substrate-free state. On the other hand, it is not advisable to use the crystal structures of CES1 bound to covalent ligands. This is because the catalytic residues, along with other residues in the catalytic binding site, are in conformations suitable for binding covalent inhibitors, rather than for accommodating non-covalent substrates.

3.1.2. CES2 homology modeling structures

The crystallographic structure of CES2 remains unresolved to this day. As a result, homology modeling is necessary to obtain the three-dimensional structure of this CES isoform in order to model the binding mode of substrates in the catalytic site of the enzyme. Different research publications have reported distinct strategies for generating the homology model structure of CES2. One of the most common methodologies involves using tools provided by the Swiss Institute of Bioinformatics server (www.expasy.org/). In the early publications, the process of generating the CES2 model followed a two-steps procedure: first, the amino acid sequence of human CES2 was retrieved from the Swiss-Prot database, and then submitted to the Swiss-Model[67] server for fully automated protein structure homology modeling[68–70]. Over time, this methodology became more automated, allowing users to directly download pre-existing CES2 models created by other researchers via the Swiss-Model server[24,71,72].

The second approach involves generating the CES2 structure using homology modeling software. Several studies have reported the use of the open-source *Modeler* software[73] to generate the homology model of CES2 and perform subsequent molecular modeling studies on this isoform[74,75].

In recent years, *AlphaFold2*, a powerful neural network-based model methodology has been developed for predicting the three-dimensional structures of proteins[76]. Since its beginning in 2021, this machine learning approach has generated more than 200 million protein structures, all freely available for download from the AlphaFold database (alphafold.ebi.ac.uk). To the best of our knowledge, despite its accessibility and remarkable accuracy, an AlphaFold-predicted human CES2 structure has not yet been utilized for the molecular modeling of this enzyme with any substrate.

3.2. Molecular modeling methods

To analyze the results from experimental enzymology studies of the CES-substrate complexes, a series of molecular modeling methodologies can be employed. These computational techniques were selected to investigate, at an atomistic level, the kinetic parameters of different substrates for both CES isoforms. This section is organized in two subsections: 1) molecular modeling approaches (molecular docking, molecular dynamics and free-energy of interaction analysis) used to study the affinity constant (K_M) and 2) hybrid QM/MM simulations employed to examine the catalytic constant (k_{cat}).

3.2.1. Molecular modeling methods to analyze affinity constant - K_M

Every molecular modeling campaign begins with a molecular docking procedure, aimed to identify substrate conformations that yield the lowest-energy interactions with residues in the CES catalytic binding site. In the subsequent stage, the CES-substrate complex is subjected to molecular dynamics (MD) simulation to characterize its dynamic behavior and stability in an explicit aqueous environment and at physiological temperature. Finally, a free-energy interaction analysis is performed based on the MD simulation data obtained for the CES-substrate complex. A small subset of studies incorporate all three of these molecular modeling methods in the investigation of CES-substrate complexes. Although a few studies incorporate all three of these molecular modeling approaches when investigating CES-substrate interactions, most rely solely on molecular docking to identify the optimal substrate conformation within the CES catalytic binding site.

The first molecular docking studies investigating the selectivity of CES1 and CES2 were conducted by the group of Vistolli et al. [68,77]. In these works, 40 known CES substrates were subjected to molecular docking protocols using two crystallographic structures of CES1 (PDB codes: 1MX9 and 1YAJ) and a homology modeling structure of CES2. Different scoring functions were calculated and correlated with the experimentally reported K_M values to develop predictive models of substrate affinity toward both CES isoforms. In both cases, the results revealed a strong correlation between K_M and the calculated lipophilic interaction scores, highlighting the central role of hydrophobic interactions, primarily attributable to the abundance of apolar residues within the catalytic binding site[68,77].

A combination of molecular docking and MD simulations have been employed to assist in the design of selective inhibitors of human CES2. These studies were focused on the design, synthesis and structure-activity relationship of glycyrrhetic acid and benzofuranone derivatives (Figure 4)[69,78]. In the first work, molecular docking was performed using the most active and selective glycyrrhetic acid derivative against both CES isoforms to elucidate its 1000-fold preference for CES2 over CES1[69]. The results indicated that this derivative displayed a greater number of hydrogen bond interactions with residues in the CES2 catalytic binding site residues than in CES1. In addition, the

conformation adopted by the inhibitor in CES1 complex was positioned farther from the catalytic serine residue than in the CES2 complex[69]. In the second study, a similar molecular docking analysis was performed for the most active and selective benzofuranone derivative against both isoforms. The results were consistent: the inhibitor exhibited more favorable interactions and a higher number binding site contacts with CES2 residues compared to CES1[78]. Furthermore, MD simulation and free-energy decomposition analyses of the CES2-inhibitor complex revealed that the complex remain stable over 50 ns of simulation and that the hydrophobic interactions played a major role in the binding of the inhibitor to CES2[78]. As shown in Figure 4, both selective CES2 inhibitors share the presence of a carboxylic acid group in their molecular structures.

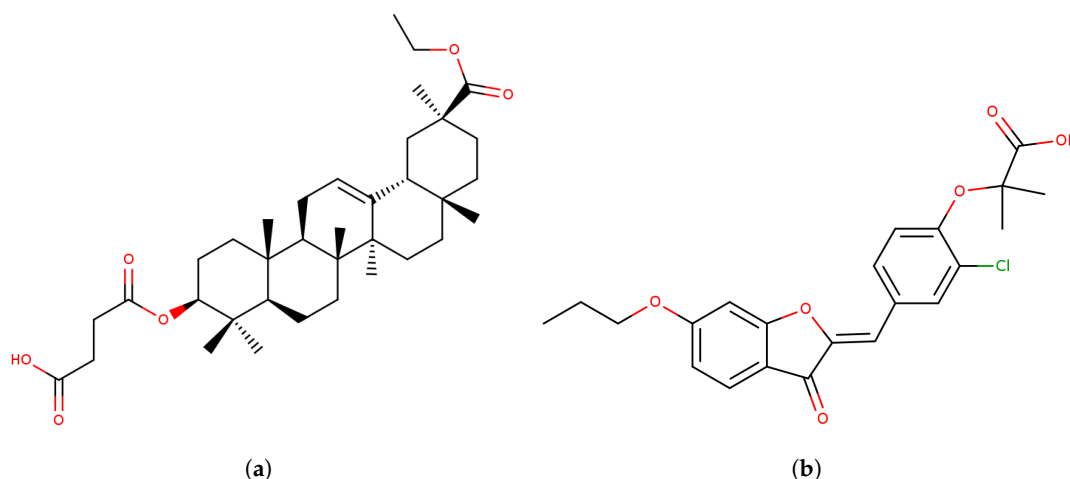


Figure 4. Molecular structure of CES2 selective inhibitors. (a) Glycyrrhetic acid derivative. (b) Benzofuranone derivative.

In a more recent study[24], molecular docking, MD simulation and free-energy interaction decomposition analyses were performed on two families of previously studied substrate families: five *p*-nitrophenyl ester derivatives[36] and two pyrethroid stereoisomers[23], using both CES1 and CES2. Consistent with earlier findings, hydrophobic interactions were found to contribute substantially to the CES-ligand free-energy of interaction, correlating well with experimentally determined affinity constants (K_M)[24]. An additional key observation was that the increase binding cavity volume of CES2, relative to CES1, enabled one pyrethroid stereoisomer to adopt a distinct interaction pattern and thereby maintain a higher affinity (lower K_M) for CES2 compared with its corresponding stereoisomer. Overall, the substrates analyzed in this work formed the most favorable interaction patterns with residues in the respective CES catalytic binding sites, resulting in the highest attainable affinity for each isoform[24].

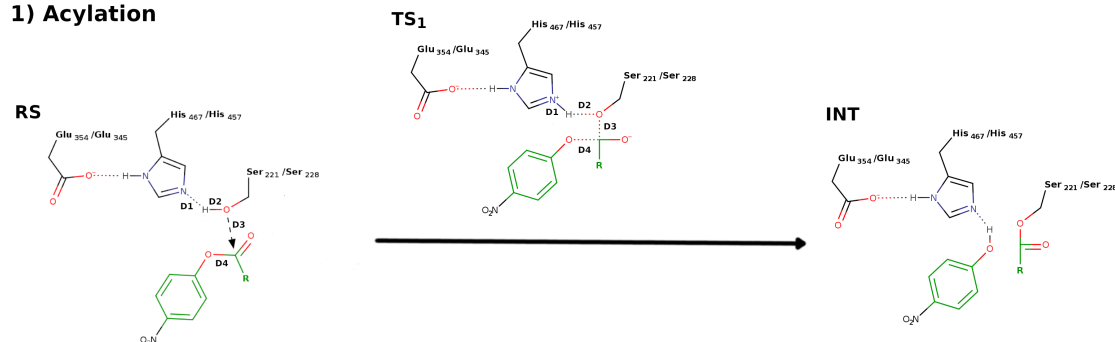
3.2.2. Hybrid QM/MM simulation to analyzed catalytic constant - k_{cat}

The study of enzyme-ligand catalytic constant through computational chemistry requires specialized methodologies to analyzed the free-energy of activation associated with the enzyme-catalyzed hydrolytic reaction. The most commonly employed approach for this purpose is hybrid QM/MM simulation. In this method, the ligand and the catalytic residues directly involved in the hydrolysis reaction are modeled using quantum mechanics (QM), while the remaining enzyme residues are described with a molecular mechanics (MM) force field.

The general hydrolysis mechanism of esterases, such as CES, is well established and involves two consecutive reaction steps. First, the acylation step, where the carbonyl carbon of the substrate is attacked by the hydroxyl moiety from the catalytic serine, leading to

the formation of an acylated serine intermediate and the release of the alkyl group of the substrate. Second, the deacylation reaction, where the carbonyl carbon of the acylated serine is attacked by the oxygen of a water molecule, yielding the carboxylic acid portion of the substrate and regenerating the free serine residue, thereby allowing a new catalytic cycle to begin (Figure 5)[3,24,37].

1) Acylation



2) Deacylation

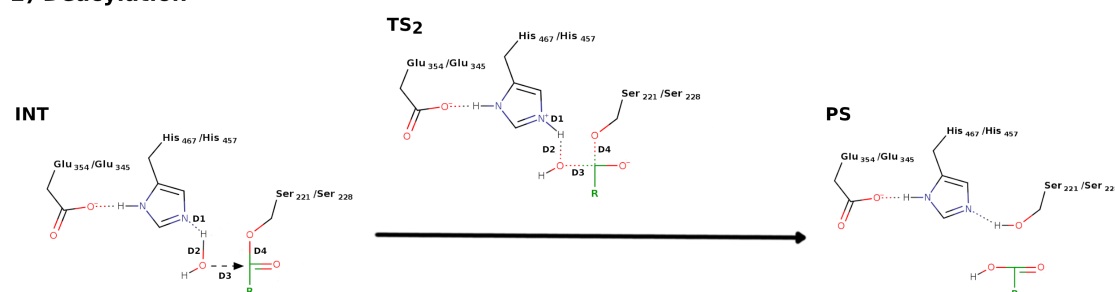


Figure 5. Acylation and deacylation reaction pathways for CES1 and CES2 catalyzed hydrolysis of the ester ligands. *p*-nitrophenyl ester derivatives were used as example. Reactant state (RS), first transition state (TS1), intermediate (INT), second transition state (TS2) and product state (PS).

There relatively few reported studies employing hybrid QM/MM simulation for substrates hydrolyzed by human CES, likely because of the greater complexity of this methodology compared with the molecular modeling methodologies described in the previous section. Given the importance of human CES in the metabolism of abuse drugs, several works have investigated the hydrolysis mechanism of cocaine by CES1 and CES2. The first study reported the hydrolysis mechanism of cocaine by CES1 using hybrid QM/MM simulation alongside the experimental determination of the corresponding kinetic parameters (K_M and k_{cat})[37]. The QM region was parameterized with the semi-empirical SCC-DFTB method, while the MM region with CHARMM27 force field. The reaction was modeled using an umbrella sampling approach, where the reaction coordinate (RC) was defined as a linear combination of four covalent bond distances: two forming and two breaking. The simulations indicated that CES1 catalyzes the hydrolysis of cocaine to benzoylecgonine and methanol (Figure 1) through a single-step acylation reaction followed by a single-step deacylation stage, each showing a distinct transition state (TS). The combined free-energy profile of both reaction steps revealed that the acylation TS is the rate-limiting reaction, with a free energy barrier of 20.1 kcal/mol. This result is in close agreement with the experimental free-energy barrier, derived from the catalytic constant, which was 21.5 kcal/mol, demonstrating the high accuracy of the hybrid QM/MM methodology employed in this study.[37].

A second article reported a similar study that the previous one, exploring the hydrolysis of cocaine by CES2 to produced ecgonine methyl ester and benzoic acid (Figure 1). In

this study, the QM region was parameterized with the semi-empirical PM6 method and the MM area with an Amber18 force field (ff99SB). An umbrella sampling method was again in this case used to follow the two steps hydrolysis reaction, exploring two covalent bond lengths, one covalent bond forming and one covalent bond breaking. The calculated 2D-PMF profiles of the full catalytic cycle showed that every reaction stage proceed with an tetrahedral intermediate structure and two TS points with the TS₄ (formation of benzoic acid) was identified as the rate-limiting step of the cocaine hydrolysis by the CES2, displaying an activation free-energy barrier of 19.5 kcal/mol. This lower energetic barrier respect to CES1 is in agreement with previous experimental results that showed a higher turnover number for CES2 than CES1.

In the previous section, a work where two families of substrates, *p*-nitrophenyl esters and pyrethroid stereoisomers, in complex with CES1 and CES2 were reported. In this reported study, two *p*-nitrophenyl ester derivatives and the two pyrethroid stereoisomers were submitted to hybrid QM/MM simulation to analyzed the hydrolysis produced by both CES isoforms[24]. The followed QM/MM simulation protocol was similar to the previous study of cocaine and CES1[37], the QM region was parameterized with the same semi-empirical SCC-DFTB3 method and the MM region with Amber20 force field (ff14SB). As with the previous example, in this case the study was also based in an umbrella sampling method, but in this study the distances of four covalent bond lengths, two covalent bonds forming and two covalent bonds breaking, were combined linearly, following a linear combination of distances (LCOD), to generate the reaction coordinate (RC). In all the modeled reactions, the hydrolysis of the substrates by both CES followed a single-step acylation stage follow by one-step deacylation stage, with one transition state (TS) for every one of these reactions. The analysis of the results showed that the rate-limiting step for the studied substrates reactions were related to the TS with the higher steric hindrance molecular structure produced during the course of the hydrolysis, acylation and deacylation stage, reactions [24]. The conclusion of this work was that the CES selectivity is not only related to the molecular size of the alkyl or acyl groups of the substrates, but a more complex scenario driven by the initial conformation of the ligand in the interaction with the CES binding site[24].

In general, all the reported studies in this section showed good correlation between the experimental catalytic constant (k_{cat}) and the calculated free-energy of activation from the rate-limiting TS reaction stage, exhibiting an accurate methodology of the hybrid QM/MM simulation studies, despite the mentioned differences in the general hydrolysis mechanisms, displaying two or four TS structures.

4. Part III: Combination of experimental and computational enzymology

This last section of the article is dedicated to the published works where both experimental and computational methodologies were combined to study the hydrolysis properties of different substrates by the different CES isoforms.

In the first one, the study of fenofibrate hydrolysis in presence of CES1 and CES2 was studied in order to identify the main enzyme responsible for its metabolization in humans[40]. The kinetic parameters were obtained by analyzing the formation rate of fenofibric acid (the hydrolytic metabolite of fenofibrate) through HPLC-UV in presence of human microsoms and recombinant CES1 and CES2 enzymes. These results exhibited that the affinity constant is slightly higher for CES1, but the main difference was present in the catalytic constant, where CES1 displayed a extensive faster reaction rate than with CES2[40]. The authors performed a molecular docking study of the substrate in the binding site of both CES isoforms. This study showed that fenofibrate exhibited a more favorable docking score into the catalytic binding site of CES1 than CES2, similar to the previous

displayed experimental affinity results. Hybrid QM/MM simulations were not performed in this publication[40]. This study would be very helpful to understand the reason behind the high difference in the hydrolysis of fenofibrate between the CES isoforms.

The second study, reported the design and CES selectivity of a family of four fluorescent substrates derivatives of naphthalimide structure [17]. The enzymatic assays were followed by measuring the emission intensity of the hydrolysis product at 520 nm (excitation 450 nm) after incubation with CES1 and CES2. Only the two derivatives containing the amide and carbamate moieties in their structures showed specific hydrolysis only in presence of CES2 (NIC-1 and NIC-2, figure 4). The other two carbamates with the nitrogen bonded to all carbon and no hydrogen atoms, showed inhibitory effects over both CES isoforms (NIC-3 and NIC-4, figure 4). In order to study the change in the enzymatic activity from CES substrate to inhibitor, NIC-4 binding properties were studied by molecular modeling methods with CES1 and compared with the known substrate *p*-nitrophenyl acetate (pNPA) [17]. After molecular docking and MD simulations, the binding free-energy of interaction analysis displayed that both NIC-4 and pNPA exhibited similar pattern of interaction with the residues of CES1 binding site. In addition, a steered MD simulation was performed, where the structures of the substrates and the binding site residues were parameterized with SCC-DFTB method and the remaining part of the enzyme with Amber16 ff14SB force field. The reaction coordinate was set as the distance from the catalytic serine oxygen to the carbonyl carbon of the substrates, representing the nucleophilic attack in the hydrolysis reaction. During the simulation of the hydrolysis reaction for pNPA with CES1, the proton from the catalytic serine residue is automatically transfer to the nitrogen atom in the catalytic histidine, following the normal acylation mechanism with an estimated energetic barrier of 20 kcal/mol. In the other hand, in the simulation of NIC-4 with CES1, such proton transfer would not happen because the methyl group near the potential nucleophilic center of NIC-4 (Figure 6) hinders the transfer path and forces the hydroxyl group of serine to orient away from the imidazole group of histidine. These molecular modeling results helped the authors to explained the mechanism of CES inhibition produced by NIC-4[17].

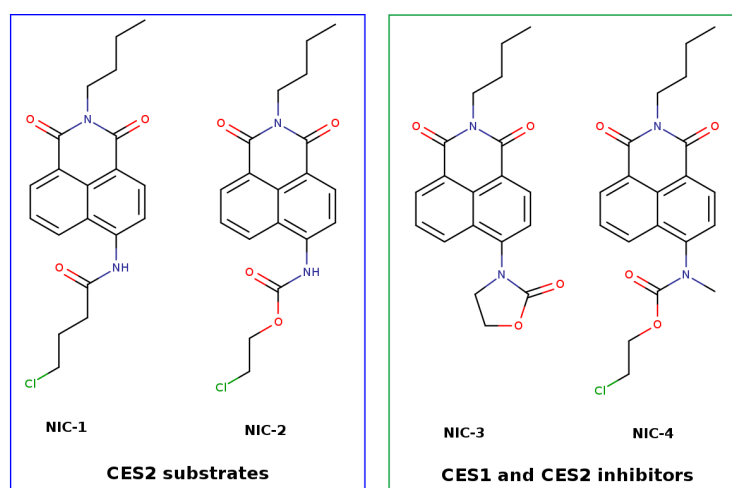


Figure 6. Structure of the fluorescent substrates derivatives of naphthalimide (NIC)[17].

5. Conclusions

This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.

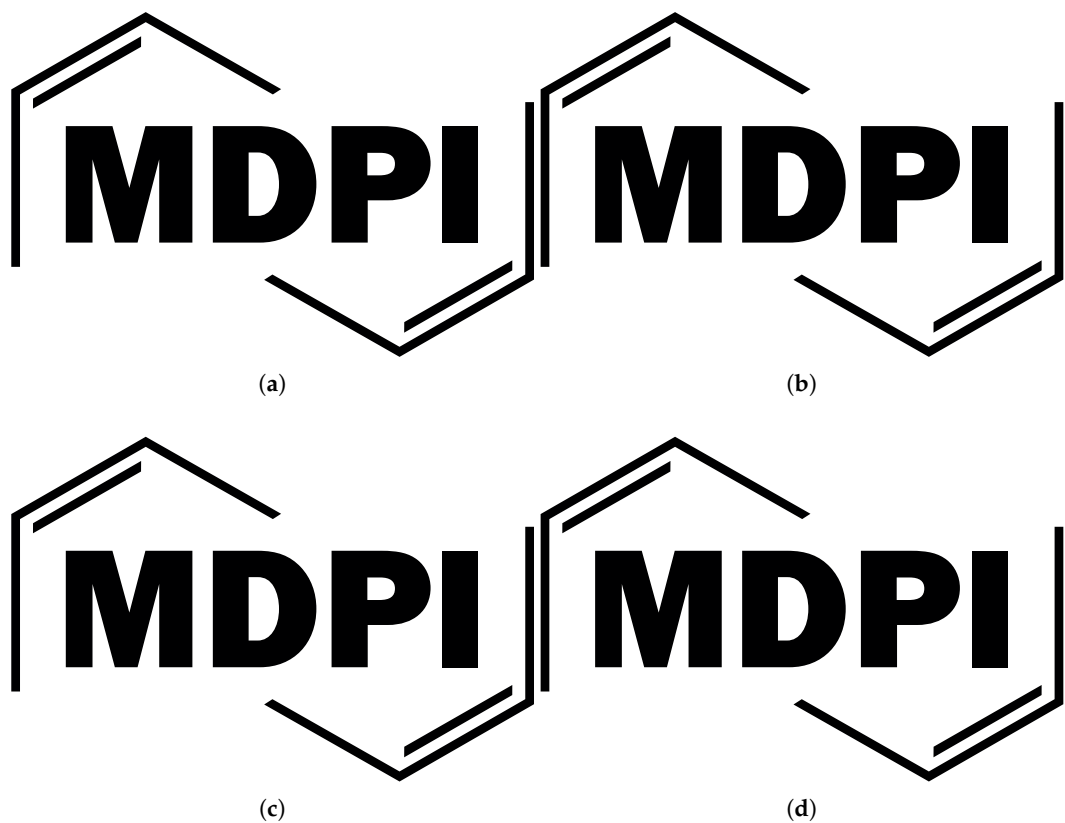


Figure 7. This is a wide figure. Schemes follow the same formatting. If there are multiple panels, they should be listed as: (a) Description of what is contained in the first panel. (b) Description of what is contained in the second panel. (c) Description of what is contained in the third panel. (d) Description of what is contained in the fourth panel. Figures should be placed in the main text near to the first time they are cited. A caption on a single line should be centered.

Table 3. This is a wide table.

Title 1	Title 2	Title 3	Title 4
Entry 1 *	Data	Data	Data
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* Tables may have a footer.

6. Discussion

Authors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

7. Conclusions

This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.

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Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three letter acronym
LD	Linear dichroism

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