CHAPTER II

LITERATURE REVIEW

2.1 Marine invertebrates and their bioactive natural products

Since last decades, a number of natural products from marine organisms such as algae, sponges, cnidarians, mollusks, tunicates, and microorganisms have been discovered. Several studies showed that marine invertebrates produced a large number of active natural products thus became new sources of natural products to study, especially marine sponges (phylum Porifera) and cnidarians (phylum Coelenterata). Many different groups of compounds such as terpenoids, polyketides, and amino acid derivatives were found in sponges and other marine invertebrates. Nowadays, researchers are still searching for new sources of natural products from marine organisms since there is enormous biodiversity under the sea (Blunt *et al.* 2011).

A group of marine invertebrates for which a number of researchers have paid attention is nudibranch. From previous studies, many secondary metabolites isolated from nudibranchs were reported and categorized into 3 main groups including terpenoids, macrolides, and alkaloids. Deoxoscalarin, a terpenoid from the nudibranch *Glossodoris sedna* showed anti-inflammatory activity in inhibiting mammalian phospholipase PLA_2 enzyme (Gavagnin *et al.* 2000). Ubapualide B, a macrolide isolated from *Hexabranchus sunguineus*, exhibited antifungal activity by inhibiting the growth of *Candida albicans* and cytotoxic activity by inhibiting proliferation of leukemia cells (Pawlik *et al.* 1988). For another compound as a member of alkaloids, tamjamine K from *Tambja ceutae*, presented cytotoxic activity on human cervical cell line (Carbone *et al.* 2010).

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2.2 Anatomy, physiology and chemical defenses of the nudibranchs

Nudibranchs are one of the most marine organisms which have been studied. They are classified in phylum Mollusca, class Gastropoda, subclass Opisthobranchia, order Nudibranchia (Miller 1996). They inhabit in the marine environment at exceedingly distant geographical regions, such as Mediterranean Sea, the north-east Allantic, the eastern Pacific, and the gulf of Thailand (Proksch 1994; Charupant *et al.* 2007; Camacho-Garcia and Gosliner 2008)

The body of nudibranchs is elongated and narrowed with various shapes and bright colors. The texture of their mantles is soft and velvety. Average life cycle takes about 6-12 months. Nudibranchs have both male and female reproductive system in same body. Although individual nudibranch contains eggs and sperms, they are not self-reproductive organism. Two nudibranchs will move alongside to construct a conjugation tube (Kasamesiri *et al.* 2012) (Figure 1). For the conjugation process which is dealing with the delivery of sperm sacs through the conjugation tube and allowing subsequent fertilization. After sperm exchange, the two nudibranchs disconnect the tube and get separated to independently lay their egg masses which are called egg ribbons containing millions of eggs.

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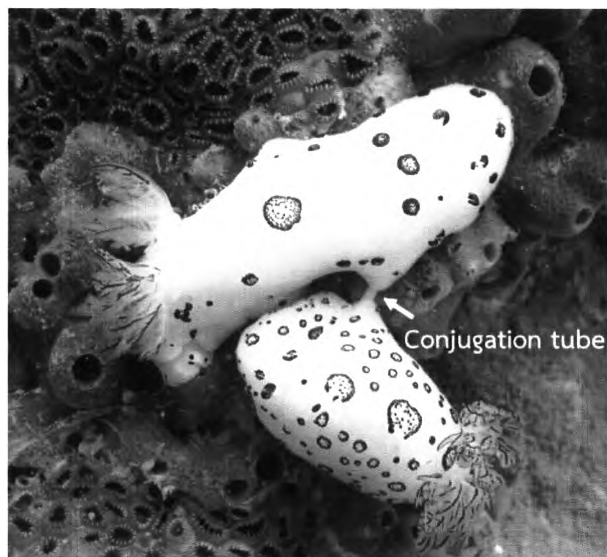

Figure 1. Conjugation process of the two nudibranchs, producing conjugation tube.

The rhinophoral sheaths, the sense organs, are located in their heads. These organs are chemical sensors which are responsible for smelling and tasting chemicals in the water leading to their food or communication with other nudibranchs. Some nudibranchs have gills as their respiratory organ while the others without gills have cerata for gas exchange (Figure 2). Particular nudibranchs have nematocysts, spicules, or small poison glands spreading on the mantle to protect themselves from predators.

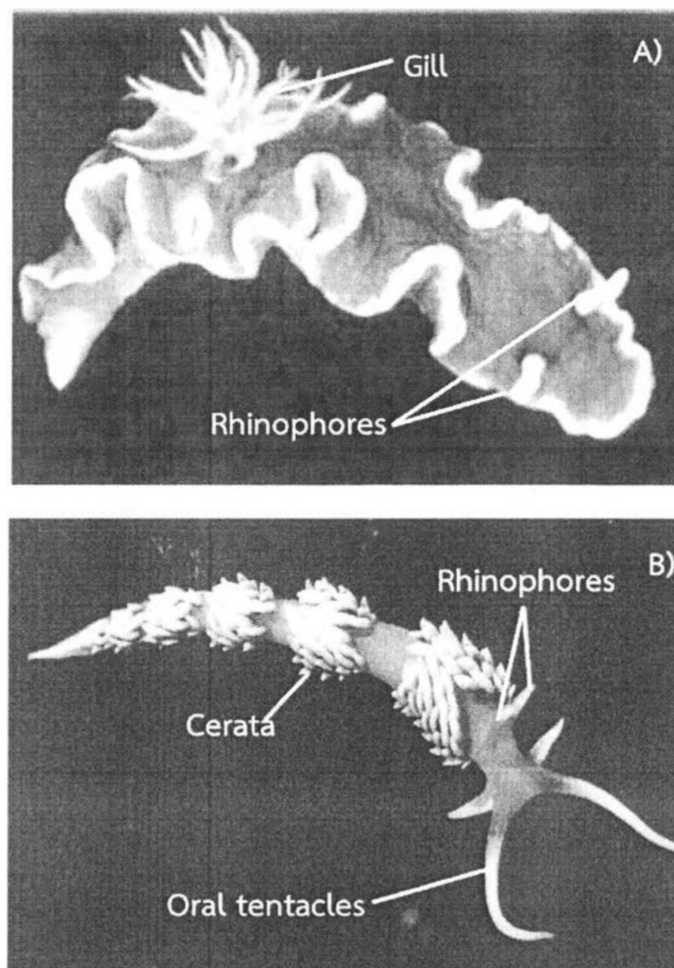

Figure 2. Physiology of nudibranch using **A)** gill or **B)** cerata as respiratory organ (Willan 1987; Rudman 1990).

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As in a subclass of gastropods, Opisthobranchia members generally lack hard shell covering their mantles. It seems that they are easily eaten by fish, crabs, or other predators. The loss of the shell is restituted by various adaptations including camouflage and accumulation of toxic chemicals in the body. Sea hares (order Anaspidea) and sacoglossans (order Sacoglassa) contain toxic chemicals obtained from their plant diets. Besides, other creatures in the same subclass such as, Cephalaspidea and Notaspidea also have evidence-based chemical defense mechanisms involving biotransformation of the food chemicals to toxic compounds. Among discovered nudibranchs, dorid (order Nudibrachia) has been paid the most attention from the chemists due to the relation of obtained secondary metabolites and their sponge diets (Pawlik 1993; Paul *et al.* 2007).

The relationships between chemical substances found in nudibranchs and their diets have been consecutively presented. It is reported that a defensive mechanism of the dorid nudibranch was the usage of chemicals as cytotoxic agents to protect itself from being preyed. This chemical defense was generated by a direct accumulation of secondary metabolites from the nudibranch diets and subsequent delivery to the predator during predation (Proksch 1994). The evidence that dorid nudibranchs stored particular metabolites was based on observation and comparison of the metabolites contents in nudibranchs and sponge diet samples. For examples, the metabolites obtained from the nudibranch *Chromodoris marislae* were related to metabolites of its sponge diet *Pleraplysilla spinifera* (Hochlowski and Faulkner 1981).

In 1988, Pawlik *et al.* studied the chemical defense of the Spanish dancer nudibranch *Hexabranchus sanguineus*, a bright colored shell-less sea slug commonly found in Indo-Pacific coral reefs (Pawlik *et al.* 1988). They found that the nudibranch derived a substance with potent cytotoxic activity from the chemical(s) of its diet sponge *Halichondria* sp. In 2007, Charupant *et al.* reported the accumulation and relation of secondary metabolites found in the nudibranch *Jorunna funebris* and its diet sponge *Xestospogia* sp. by which it was eaten (Charupant *et al.* 2007). The assumption for the function of sponge-related metabolites on nudibranch self-protection was verified by a localization study. The secondary metabolites from

different parts of the nudibranch were determined. Renieramycins and jorunnamycins were localized in body parts and egg ribbons rather than the digestive system of the nudibranchs. This finding suggested that these compounds might play a role in the defensive mechanism to protect the animal's life and developing eggs.

2.3 *Bis*-tetrahydroisoquinoline alkaloids from marine organisms

Several isoquinoline alkaloids were isolated from marine organisms such as sponges, tunicates, and nudibranchs and showed their potent cytotoxic activity on many cancer cell lines (Suwanborirux *et al.* 2003; Mayer and Gustafson 2006; Charupant *et al.* 2007). Structures of *bis*-tetrahydroisoquinoline alkaloids contain two fused moieties of tetrahydroisoquinolines. The examples of bioactive compounds for this structure type are saframycins, renieramycins, jorumycins, and jorunnamycins (Figure 3). *Streptomyces lavendulae* is the marine bacterium producing saframycin A, a member of saframycins, which exhibited anti-proliferative activity against various tumor cell lines (Ishiguro *et al.* 1978). Saframycin Mx1 from the bacterium *Myxococcus xanthus*. was reported as an antitumor antibiotic agent (Scott and Williams 2002). Cytotoxic renieramycins were found in the sponge *Xestospongia* sp. which inhabits in the gulf of Thailand (Suwanborirux *et al.* 2003) and also found in the specimens of the nudibranch *Jorunna funebris* (Charupant *et al.* 2007). Jorumycin as unstable pale-yellow powder was first isolated from *J. funebris* specimens collected from the Pacific ocean (Fontana *et al.* 2000). Although jorumycin showed its unstable property, the stabilized jorunnamycins A, B, and C in the extract of *J. funebris* specimen after pretreated with potassium cyanide (KCN), were obtained in a large quantity (Charupant *et al.* 2007).

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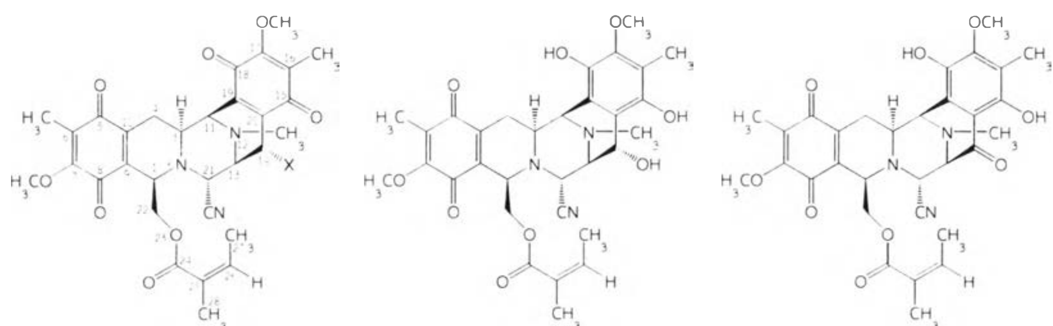
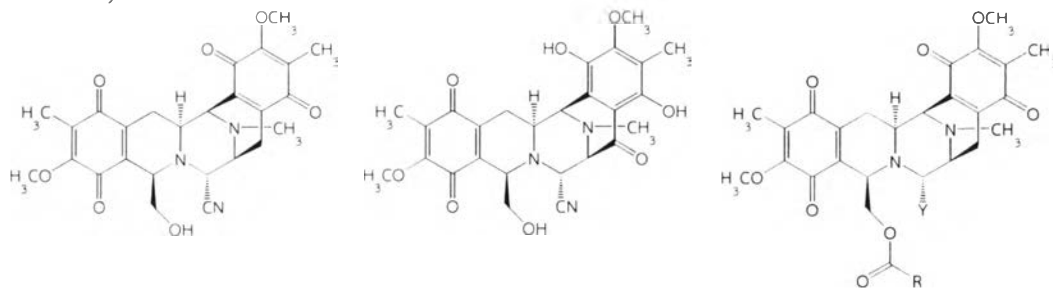


Renieramycin M: X=H
Renieramycin O: X=OH

Renieramycin N

Renieramycin Q



Jorunnamycin A

Jorunnamycin B

Jorunnamycin C: R=C_2H_5, Y=CN
Jorumycin: R=CH_3, Y=OH

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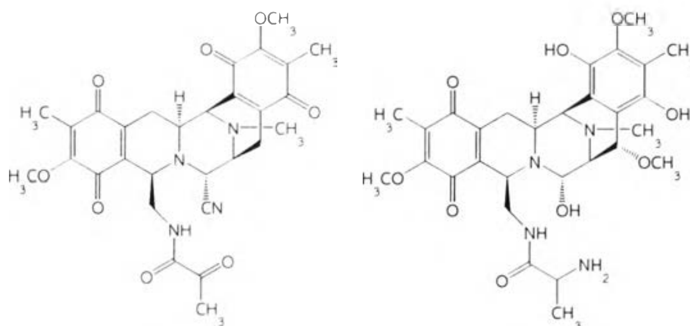

Saframycin A

Saframycin Mx1

Figure 3. Structures of representative *bis*-tetrahydroisoquinoline alkaloids. (The numbering on the core structure of renieramycin M can be used for all core structures of the *bis*-tetrahydroisoquinoline groups.)

2.4 Jorunnamycin A as an intermediate of renieramycin M-jorunnamycin A derivatives.

The nudibranch, *J. funebris* has been found in the gulf of Thailand, Chonburi province, Thailand. From observation, *J. funebris* was always found to eat the blue sponge *Xestospogia* sp. which accumulated renieramycin M (RM) as a major metabolite. Both organisms contain renieramycin M with an impressive quantity and other renieramycins including renieramycin N (RN), renieramycin O (RO), renieramycin Q (RQ) with diminutive yields. The interesting thing is that jorunnamycins including jorunnamycin A, jorunnamycin B and jorunnamycin C, were isolated only from the nudibranch but not from the blue sponge diets. These two groups of compounds (renieramycins and jorunnamycins) share their core structure similarity and both are classified as members of *bis*-tetrahydroisoquinoline alkaloids. These two groups showed cytotoxic activity to several human cancer cell lines (Table 1) (Charupant *et al.* 2007).

Table 1. Cytotoxicity of jorunnamycins A-C and renieramycin M to cancer cell lines (Charupant *et al.* 2007).

Compound	IC_{50} (nM)	
	HCT116	QC50
Jorunnamycin A	13.0	59.0
Jorunnamycin B	445.0	618.0
Jorunnamycin C	1.5	2.8
Renieranycin M	7.9	19.0

HCT116=human colon carcinoma; QG56 human lung carcinoma

There was a study attempting to find new potent *bis*-tetrahydroisoquinoline-type anticancer agents by using renieramycin M as a prototype to semi-synthesize new compounds (Saito *et al.* 2004). These semi-synthetic compounds were modified by changing the side chain group at position C-22 to obtain 22-*O*-acyl derivatives. Some derivatives were reported for more potent cytotoxic activity to human colon cancer cell lines as compared to their prototype (Charupant *et al.* 2009). The ester bond incorporating the angelate part at C-22 had to be cleaved from the core

structure of renieramycin M resulting in the production of jorunnamycin A containing a free alcohol moiety. Unfortunately, normal acid/alkaline hydrolysis was unable to cleave this ester bond. The process of jorunnamycin A preparation deals with the three-step reaction involving hydrogenation, hydride reduction and oxidation for this specific chemical transformation (Figure 4). Surprisingly, jorunnamycin A was also found in the nudibranch *J. funebris*. As previously mentioned, *J. funebris* is a sponge-feeding animal which specifically fed on the sponge *Xestospongia* sp. Since jorunnamycin A, the deangeloyl renieramycin M, was found in the nudibranch but not in the sponge *Xestospongia* sp., leading to a hypothesis that the reaction involved in the conversion of renieramycin M to jorunnamycin A is catalyzed by specific enzyme(s) in the nudibranch tissues. Considering the structure of these two alkaloids, the enzyme(s) involved in the hydrolysis of the ester bond at C-22 is possibly an esterase type.

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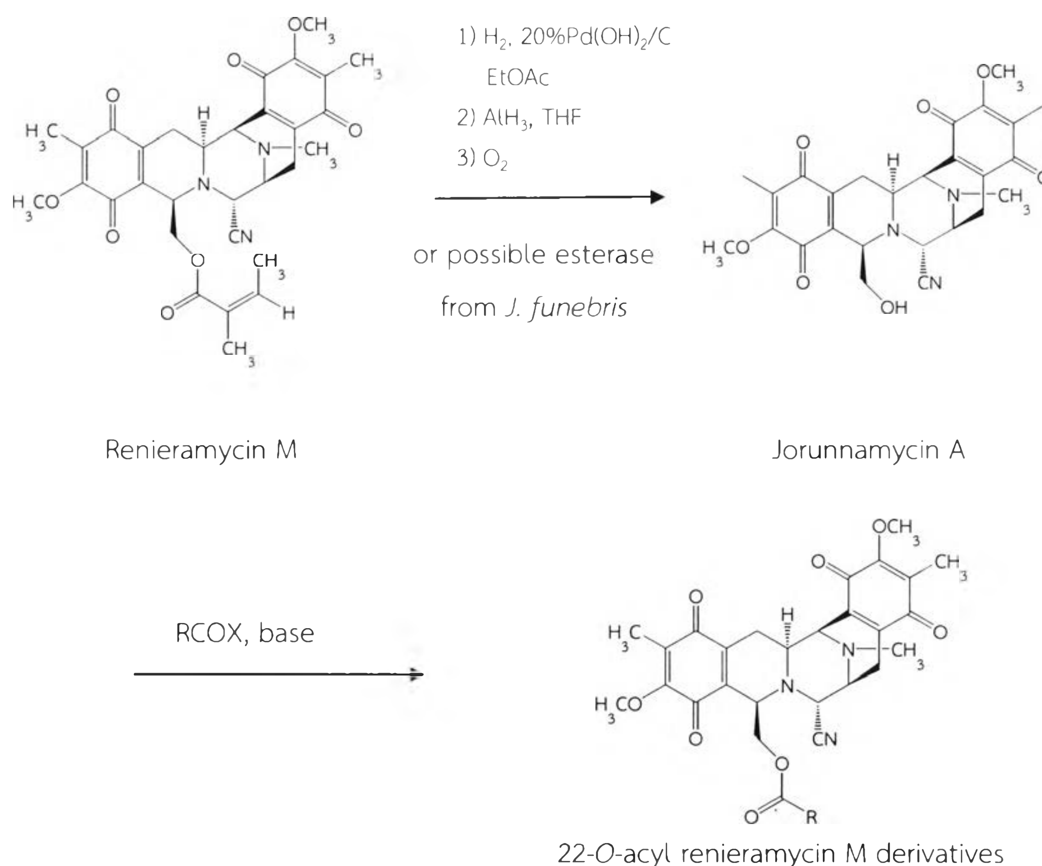

Figure 4. Steps in preparation of 22-*O*-acyl renieramycin M derivatives.

2.5 Esterase enzymes

Esterase is a group of enzyme classified in the hydrolase class of which the enzymes show very wide substrate specificities such as amides, peptides, esters, and triglycerides. Esterases have played roles in catalyzing the hydrolysis reaction of the ester bond giving acid and alcohol final products (Figure 5).

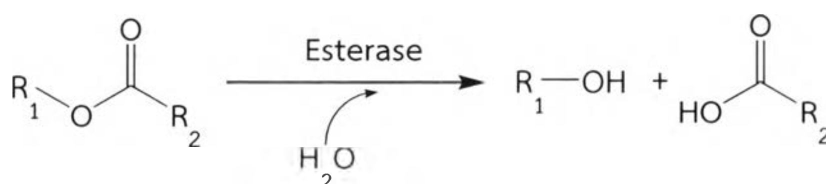

Figure 5. Hydrolysis equation of catalytic reaction by esterase.

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A reaction by esterase works by a catalytic triad of the enzyme which recruits a particular set of amino acids forming a pocket with a three-dimensional shape driving biotransformation of the substrate to the following intermediate(s) or product(s). The mechanism of the esterase is to catalyze the reaction of ester linkage cleavage by the addition of a water molecule to the substrate. The catalytic triads of esterases commonly contain three amino acids including glutamic acid (Glu) or aspartic acid (Asp), histidine (His), and serine (Ser) (Dodson and Wlodawer 1998). The acid residue (Glu/Asp) induces the basic residue (His) to act as a stronger base and generates deprotonated serine. The serine amino acid serves as a nucleophile and forms complex with the substrate giving a tetrahedral intermediate. Once the ester bond is cleaved, the alcohol product leaves the pocket while the rest of the molecule containing acid part remains at the binding site to yield a resulting complex called acylated enzyme. Then the water molecule as the nucleophile will interact with the acylated enzyme and the bond is subsequently broken to generate the acid product (Montella *et al.* 2012) (Figure 6).

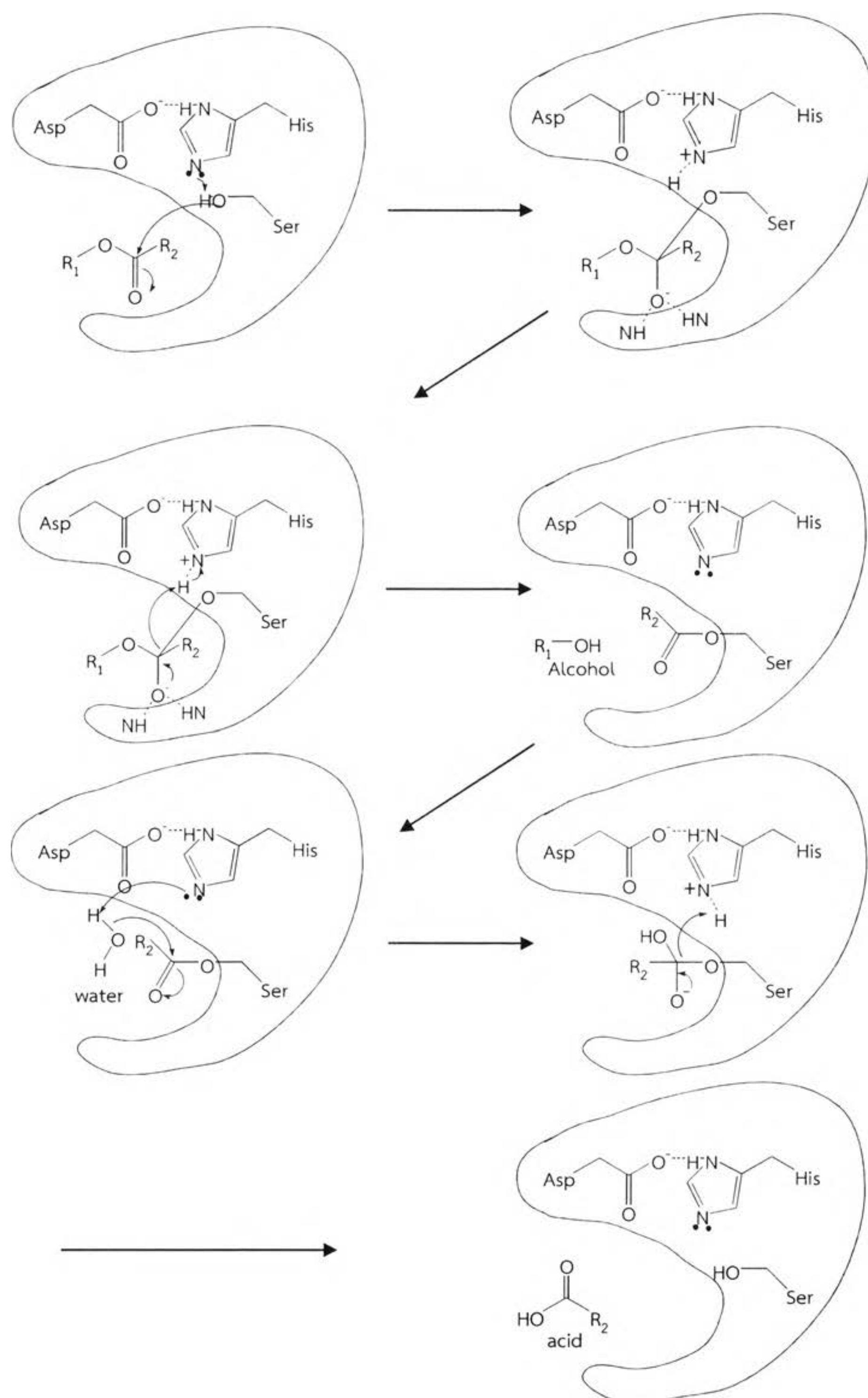

Figure 6. Mechanism of hydrolysis reaction by an esterase containing Asp/His/Ser catalytic triad (Montella *et al.* 2012).

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There are many systems used to categorize the esterase enzymes. The most common way is to consider the type of ester bonds in the substrate molecules subjected to the enzymes; for example, carboxylesterase, thioesterase and phosphoesterase. For more sophisticated consideration, the conserve motif of amino acid sequences is currently used for enzyme classification. For example, the carboxylesterases mostly have the sequence of glycine-X-serine-X-glycine; X stands for any amino acid presented in the conserve motif of the amino acid sequence (Akoh *et al.* 2004).

Esterase is one of the most important enzymes for living organisms. These enzymes can be found in all parts of the animal body. For example, acetylcholinesterase plays the role in degradation of the acetylcholine, a neurotransmitter in the brain. Lipase from pancreas degrades the lipid molecule in small intestine for lipid absorption. In the blood circulation system, leucocyte esterase, responsible for degradation of leucocyte, is found and used for detection of the infection abnormality. There was a report about the distribution of enzymes employed in biotechnological industries (Panda and Gowrishankar 2005). Sixty-five percent of enzymes used in industry were hydrolases and thirty-eight percent of the used hydrolases were esterases. Presently, esterases are widely used in degradation of natural materials and industrial chemicals. For example, feruloyl and cinnamoyl esterases from *Asperagillus niger* can release hydroxyl cinnamic acids from wheat bran, rice bran, sugar cane and etc to use in food industry. An esterase from *Fusarium oxysporum* is used in producing flavoring and fragrance compounds from fatty acid (Panda and Gowrishankar 2005). Lipase, the carboxylesterase, from a marine microorganism *Candida antarctica* was found to have the ability to catalyze the reaction involving the conversion of degummed soybean oil to biodiesel oil (Watanabe *et al.* 2002). The extracellular lipase from *Staphylococcus epidermidis* was used in lipid synthesis processing (Joseph *et al.* 2006). In the field of food industry, the esterases including lipases were used in food processing and synthesis of flavoring and ordoring agents. Since the production of food needs to carry out in low temperature, psychrozymes, the cold adapted enzymes from psychrotrophic bacterium *Pseudomonas* sp. and molds involving *Rhizopus* sp. and *Mucor* sp., were used in milk and dairy products industry (Coenen *et al.* 1997). For the household applications, esterases seem to commonly apply as biodetergents. Lipase from a microbe *Pseudomonas* sp. is immobilized on surfaces to facilitate the removal of

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lipid stains from fabric (Hasan *et al.* 2006). *Candida antarctica* lipase was developed for using in detergent formulation (Joseph *et al.* 2008). In other industrial fields and agriculture fields, the enzymes from bacteria are used as biopesticides. Phosphotriesterase from *Pseudomonas monteilli* have been used in degradation of oganophosphorous compounds (Home *et al.* 2002). The examples of industrial applications of these enzymes used are shown in Table 2.

Table 2. Indrustrial applications of esterases (Joseph *et al.* 2008).

Fields of applications	Purpose
Medical and pharmaceutical application	Synthesis of arylaliphatic glycolipids
	Ethyl esterification of docosahexaenoic acid to ethyl-docosahexaenolate
	Synthesis of citronellol laurate from citronellol and lauric acid
Fine chemical synthesis	Optically active ester synthesis
	Ester synthesis, desymmetrization and production of peracids
	Organic synthesis of chiral intermediates
	Synthesis of butyl caprylate in n-haptane
	Synthesis of butyl lactate by transesterification
	Synthesis of amides
Food industry	Protein polymerization and gelling in fish, improvement in texture, flavor modification
	Production of fatty acids and inter esterification for cosmetics
	Conversion of degummed soybean oil to biodiesel fuel
	Synthesis of lipase-catalyzed biodiesel
Environmental application	Degradation of lipid wastes
	Bioremediation and bioaugumentation
	Removal of solid and water pollution by hydrocarbons, oils, and lipids

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2.6 Green chemistry by enzymatic transformation as an alternative way of environment-friendly synthesize chemicals

The enormous pool of biodiversity is found in marine ecosystem. Marine environment has covered approximately 70% of surface on the earth. Because of the wide range of conditions, organisms have to survive by adaptation to all extreme marine environments; for example, production of cold-adapted enzymes (Al Khudary *et al.* 2010). The modifications for subsistence of these organisms lead to many studies related to novel biocatalysts. Marine microorganisms have attracted many researchers to explore them since they have to adapt themselves to survive under unusual environment conditions such as high or low temperature, alkaline or acid water and hazardous environment.

For biotechnological applications, marine organisms are used for making or modifying products and can be categorized into five domains; chemistry, pharmacology, food packaging, cosmetics, agriculture and industrial applications. Some evidence reported that the enzymes from marine organisms were used as biocatalysts which presented diversity of chemical substrates.

In Pharmaceutical biotechnology field, the biocatalysts were used for enantio-selective application. There was a study of esterase enzyme from bacterium *Yarrowia lipolytica* CL180 used for separating levofloxacin from ofloxacin racemic mixture which are normally used as gram-negative antibiotics for treatment of infection in gastrointestinal and urinary tracts. Since the levofloxacin (*S*-isomer of ofloxacin) has four times potency more than *R*-isomer ofloxacin, the demand of levofloxacin is increased. Although the general process to isolate individual compound from this racemic mixture was not simply achieved, a particular esterase responsible for separating was very useful in this case (Kim *et al.* 2007).

Another example is the enantio-selective esterification by a novel thermophilic esterase (APE1547) for ibuprofen. Ibuprofen is widely used as a non-steroidal anti-inflammatory drug with an asymmetric carbon in the second position

giving (*S*)- and (*R*)-ibuprofen stereochemistry. According to their potencies, (*S*)-ibuprofen exhibited 160 folds more potent than the (*R*)-enantiomer. Therefore, the (*S*)-ibuprofen has been paid more attention for a specific isolation. The esterase APE1547 from the archaeon *Aeropyrum pernix* K1 was used to catalyze the enantio-selective transesterification of 2-octanol in ibuprofen synthesis and it seemed to have a successful efficiency for producing selected (*S*)-enantiontiomer ibuprofen (Zhao *et al.* 2011).

In addition, with the enantio-selective properties of esterases, the enzymes were used for obtaining chiral building blocks as intermediates in asymmetric syntheses. Pig liver esterase (PLE) was being most widely used in many processes such as the synthesis of an antiulcerogenic (+)-cassiol and the preparation of a subunit of the fungicidal macrolide soraphen A (Davis and Boyer 2001).

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The use of enzyme for synthesizing chemical product(s) is possibly called green chemistry since it reduces or eliminates hazardous substances in the reaction. This alternative way of using the enzyme instead of strong chemical reagent(s) is more beneficial and safe to environment and human being. Additionally, the enzyme used for the reaction is mostly able to be reused, resulting in cost savings for production