GREAT LAKES FISHERY COMMISSION

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Purification and characterization of anti-microbial bioactive peptides from sea lamprey

by:

Sang-Seon Yun[2], Weiming Li[2]

[2] Department of Fisheries and Wildlife
Michigan State University
East Lansing, MI 48823

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Abstract

In fish, it is well established that the innate immunity plays an important role in defense. Antimicrobial peptides have been isolated and characterized from several species of fish. We made an attempt to isolate some antimicrobial peptides from sea lamprey blood cells after challenge with some bacterial strains. An acetic acid extraction of blood cells from challenged fish were subjected to a series of separation methods such as solid phase extraction, cation-exchange chromatography, gel filtration chromatography and reversed phase high performance liquid chromatography combined with the antimicrobial assay. Surprisingly, the substance in the antimicrobial fractions turned out to be an aminosterol, called squalamine , originally identified from the dogfish shark. Further chromatographic and mass spectrometry data confirmed that the active fraction from the sea lamprey blood cells was identical to squalamine. This compound has been known to have anti-angiogenic property and been being developed as a cancer therapeutic agent. It can be postulated that squalmine may have an important role in defending against microbial invasion as part of innate immune system. Furthermore, it is very interesting to find a compound known to be present in other lineage of fish. This finding may indicate that squalamine is originated from an ancient fish species, sea lamprey and that this category of biomolecules is widespread throughout the vertebrates.

Introduction

It has been well established that innate immunity plays an important role in defense against bacterial and viral invasion and can be mediated by cellular and humoral elements (Beutler, 2004). The cellular components of innate immunity include macrophages and neutrophils while the humoral elements are represented by complement, lysozyme, lactoferrin and low molecular weight antibiotic substances such as peptides, lipids and alkaloids (Beutler, 2004; Boman, 1991; Daly et al., 1987; Kabara et al., 1977). In fish, there have been numerous reports regarding effector molecules believed to be involved in innate immunity and antimicrobial peptides have been among the most characterized substances in fish, including pardaxin from the moses sole fish *Pardachirus marmoratus* (Oren and Shai, 1996), chrysophsin from the red sea bream *Crhysophrys major* (Iijima et al., 2003), pleurocidin from the winter flounder *Pleuronectes americanus* (Cole et al.,1997), parasin I from the catfish *Parasilurus asotus* (Park et al, 1998), , misgurin from the loach *Misgurnus anguillicaudatus*, (Park et al, 1995), hipposin from the Atlantic halibut *Hippoglossus hippoglossus* (Birkemo et al, 2003), bass hepsidin, piscidins, and moronecidin from the hybrid striped bass (Lauth et al, 2002; Shike et al, 2002; Silphaduang and Noga, 2001).

The sea lamprey, *Petromyzon marinus*, is an ancestral jawless fish and has a unique life history with parasitic stage and migration. It is well established that this group of fish (cyclostomes) has not developed the adaptive immunity that is found only in cartilaginous fish and higher vertebrates (Fujita et al., 2004).

As a result, it is most likely that this group of fish greatly relies on innate immune system and has developed a series of molecular weapons in defense of pathogenic invasion. However, very little is known about the effector molecules involved in innate immunity in the sea lamprey. The purpose of this study was to identify antibiotic substances, mostly antimicrobial peptides, which may play a role in the innate immune defense.

In this study we focused on identifying novel antimicrobial peptides using a series of separation techniques combined with antimicrobial assays. However, surprisingly it turned out that the antimicrobial substance from blood cells was not a peptide but squalamine, an aminosterol originally isolated from the dogfish shark. Our finding indicates that the evolutionary origin of this aminosterol dates back to early vertebrate evolution.

Materials and Methods

Chemicals and equipments

Ammonium acetate (NH_4OAc), acetic acid, o-Phthaldialdyhyde (OPA), ninhydrin, mercaptoethanol, ethyl acetate and sodium chloride were purchased from Sigma (St. Louis, MO). HPLC grade solvents, acetonitrile (ACN), methanol, and trifluoroacetic acid (TFA), were from EMD Chemicals (Gibbstown, NJ), Fisher Scientific (Pittsburgh, PA) and Pierce (Rockford, IL), respectively. Ion exchange, gel filtration, and reverse phase liquid chromatography were done on Waters 600 delivery system (Waters, Milford, MA), equipped with a 996 PDA detector and a fraction collector (Waters). Polycarbonate filter papers of 1 µm and 0.45 µm pore size were from Millipore (Bellerica, MA). The fluorescence was detected using a Waters 474 detector (Waters). The centrifuges used were Allegra 6R (Beckman Coulter, Fullerton, CA) and KR 22i (Jouan Inc., Winchester, VA). Bacterial strains, *Micrococcus luteus* and *E. coli* D31 were a kind gift from Dr. X. Lauth, University of California, San Diego.

Animals

Parasitic sea lamprey were obtained from the Lake Huron by the staff of Hammond Bay Biological Station, USGS. The lamprey were transported to the wetlab facility at Michigan State University and kept in a 100 L flow-through tank at 12 °C. After acclimation, the lamprey were injected with 10^7 cells of log phase *M. luteus* and *E. coli*. 18 h after the bacterial challenge, blood was drawn using a heparinated syringe. The pooled blood was centrifuged at 3000 rpm using

Allegra 6R centrifuge at 4 °C for 20 min. The pellet was frozen in liquid nitrogen and kept at -80 until use.

Extraction

The pellet was powdered under liquid nitrogen and subjected to a 10 % acetic acid extraction. The ground blood cells were extracted in 10% acetic acid solution with cocktail of protease inhibitors (Roche, Indianapolis, IN) on ice for 3 h while shaking. After centrifugation at 20,000 x g on KR 22i centrifuge, the supernatant was filtered through a 1 µm and 0.45 µm filter papers before solid phase extraction. The filtered extract was loaded on to a 35 CC C18 Sep-Pak (Waters) that was primed with 50 ml of methanol and 10% acetic acid. After washing with water, the trapped material was eluted with 30, 50, and 80% acetonitrile (ACN) in 0.1 % trifluoroacetic acid (TFA). The eluates were lyophilized using a freeze dryer (Labconco, Kansas City, MO) and tested on bacterial lawn assay.

Chromatography

Cation Exchange Chromatography

Positive eluate (50% ACN eluate) was further separated using a HiPrep Sepharose SP column, a sulfopropyl strong cation exchange column (16x100 mm, Amersham, Piscataway, NJ). The 50 % ACN eluate was reconstituted in solvent A (20 mM ammonium acetate (NH_4OAc), pH 5.5), and loaded on to the

cation exchange column. The elution was performed with a linear gradient of 0-100 % of solvent B (2N NaCl in 20 mM NH_4OAc) for 120 min at a flow rate of 1 ml/min. 4 ml fractions were collected using a fraction collector (Waters) and the individual fractions were desalted using a Sep-Pak plus cartridge (Waters) by eluting with 5 ml of 70% ACN/0.1% TFA. UV absorption was monitored at 280 nm using a 996 PDA detector (Waters). The desalted fractions were freeze dried and tested for antimicrobial activity using the bacterial lawn assay.

Gel Filtration Chromatography (GFC)

Positive fractions from cation exchange chromatography were further separated on a TSK gel G2000 SW column (300x7.5 mm, Tosoh, Japan). The antimicrobial substance was eluted with 30% ACN/0.1% TFA at a flow rate of 0.5 ml/min for 60 min with UV absorption monitored at 214 nm.

Reverse phase HPLC

Final step of purification was done by reverse phased HPLC. The positive fractions from GFC was loaded on to a C18 Jupiter column (4.6 x 250 mm, 300 A, Pheonomenex, Macciesfield, Cheshire, UK) and eluted with a linear gradient of 20-50% ACN/ 0.1% TFA for 50 min.

Derivatiztion of squlamine with o-phthaldialdyhyde (OPA)

Derivatization of active fraction and squalamine standard with OPA was performed according to Joseph and Davies (1983). Briefly, 27 mg of OPA was

dissolved in 0.5 ml of ethanol and 5 ml of 0.4 M boric acid, pH 9.5, followed by 20 µl of mercaptoethanol. 50 µl of sample solution was mixed with 4 volumes of OPA reagent. After 2 min reaction, a 20 µl aliquot was analyzed on a C18 column. The elution of derivatized squalamine was monitored with excitation at 340 nm and emission at 455 nm using a Waters 474 fluorescent detector (Waters).

Thin layer chromatography (TLC)

Thin layer chromatography was performed on a silica plate (5 x 20 xm, Whatman, Clifton, NJ) by loading the reference and active fraction. The plate was developed with a mixture of ethyl acetate:methanol:ammonium hydroxide (5:1:1, v/v) for 1 h. After baking the plate at 90 C for 30 min, the plate was visualized by spraying 0.3 % ninhydrin solution in ethanol and heating at 90 C.

Assay of antibacterial activity

Antimicrobial assay was performed as described by Zasloff (1987). Fractions were desalted or lyophilized and then resuspended in distilled water. An aliquot of the fractions was spotted on to a lawn of *E. coli* D31 and *M. luteus* on an LB medium plate containing 1.5 % agar and incubated at 37 C for 18-24 h. Fractions displaying a microbicidal zone of clearing were pooled and subjected to the next step separation.

Mass spectrometry

Fast atom bombardment mass spectrometry (FAB MS) was performed on the active fraction. Mass spectra were obtained using a JEOL HX-110 double-focusing Fast Atom Bombardment (FAB) mass spectrometer (JEOL, Peabody, MA, USA), operable in either the positive ion mode. Ions were produced by bombardment with a beam of Xe atoms (6 keV). The accelerating voltage was 10 kV and the resolution was set at 3000. For FAB MS/MS, helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the parent ion by 50%. Shrader TSS 2000 data system generated linked scans at a constant ratio of magnetic to electrical fields (B/E). FAB MS was done at the NIH MS facility at MSU.

Results

Purification of antimicrobial substance from blood cells

Acid extracted homogenate of blood cells were subjected to solid phase extraction using C18 Sep-Pak. Antimicrobial assay of eluates from SPE revealed that the fraction eluted with 50% ACN/0.1 % TFA was most active in inhibiting the growth of bacterial strains, E. coli D31 and M. luteus. This fraction was further fractionated on cation exchange column and antimicrobial activity was found in the fractions eluting between 75 – 105 min (Fig. 1). The pooled fraction was loaded on to a GFC column and eluted with 30 % ACN/0.1% TFA. Fractions eluted between 23 – 27 min showed strong antimicrobial activity (Fig. 2). As a final purification step, reverse phase HPLC was performed. Fractions eluted at 47-50 min contained antimicrobial activity to *M. luteus* (Fig. 3).

Mass spectrometry analyses of active fraction

FAB MS analyses of the active fraction from the final HPLC step revealed an ionized peak at *m/z* 628 (positive mode). Further MS/MS analyses of ionized peak at *m/z* 628 resulted in a fragmentation pattern as in Fig 4. Major daughter ions were seen at *m/z* 548 and at *m/z* 530, indicating fragmentation of sulfate (-80) and water (-18). Database search for the compound matching the mass and the fragmentation pattern revealed that this is most likely to be squalamine, an aminosterol originally found in the dogfish shark, *Squalus acanthias* (Moore et al., 1993). The molecular structure of this compound is shown in Fig. 5.

Confirmation of the chemical structure

To verify the chemical structure of the antimicrobial factor from the sea lamprey as squalamine, a series of analysis was performed. Squalamine lactate (kind gift from Prof. Zasloff) and the active HPLC fraction were subjected to OPA derivatization followed by separation on a C18 column. Both compounds coeluted as seen in Fig. 6, which confirms the identical chromatographic properties of the both compounds. Further, thin layer chromatography was also performed to find a comigration of both compounds in a silica gel plate (data not shown). Due to the difficulties associated with getting enough material, no NMR data were obtained.

Discussion

We have identified an antimicrobial aminosterol, squalamine from the sea lamprey. It was very intriguing to find this compound from the sea lamprey since no aminosterol with antimicrobial activity has been identified from any other vertebrate species than the dogfish shark (Moore et al., 1993). It makes sense to share squalamine between the two species in the context of evolution because both fish species belong to early vertebrates with the sea lamprey most ancient. This cationic aminosterol is a spermidine conjugate of a sulfated bile acid and has very similar molecular structures to some of bile acids known for larval and sex pheromones in the sea lamprey (Li et al., 1995; Li et al., 2002, Yun et al., 2003). Bile acids are known to play an important role in digestion and lipid metabolism, especially for removal of cholesterol (Hofmann and Mysels, 1988). However, in the sea lamprey, most of known bile acids seemed to be involved in chemical communications between conspecifics. For example, larval sea lamprey uses PZS and ACA to guide the migration of adult to the streams, while 3kPZS and 3kACA were somewhat involved in chemical signaling between mature sea lamprey for spawning (Bjerselius et al., 2000; Li et al., 1995; Li et al., 2002, Yun et al., 2003). All bile acids known so far in the sea lamprey have C24 with 5 alpha configuration. In contrast, squalmaine has C27 with sulfate at C24 and spermidine at C3.

Although it is not clear yet whether squalamine is produced as responses to bacterial challenge or not, it seems obvious that this compound may play an important role as the first line of defense because it can inhibit the growth of

Gram-negative and Gram–positive bacterial strains. This cationic aminosterol is believed to work by disrupting bacterial membrane as other cationic antimicrobial peptides do (Savage et al., 2002). Previous studies demonstrated that squalamine is more potent than antibiotic substances with minimal inhibitory concentration (MIC) value of 1-10 μg/ml for microorganisms tested (Moore et al., 1993). Therefore it is likely that squalamine may provide an important defense mechanism in the sea lamprey.

In the present study, squalamine was isolated from blood cells in contrast to its initial identification from the stomach of the dogfish shark, *S. acanthias.* Since the extraction was performed on centrifuged pellet containing various cell types, it is not known what cell type was the origin of this compound. However, it is most likely that leukocytes carry this compound as a weapon against microbial invasion. Recent identification of an antimicrobial factor with molecular weight of 655 Da from channel catfish leukocytes indicates that fish leukocytes can be a carrier of antimicrobial factors (Ourth and Chung, 2004). In addition to its biological function as an effector of innate immune system, it can be speculated that squalamine may have a regulatory role by interacting with other immune cells.

Since its first identification from the dogfish shark, in addition to its antimicrobial activity, squalamine has been proven to be anti-angiogenic (Sills et al., 1998; Teicher et al., 1998; Schiller and Bittner, 1999) and as a result, has been being developed as an anti-cancer therapeutic agent (Bhargava et al., 2001; Hao et al., 2003). However, actual mechanisms that squalamine exerts its

anti-angiogenic effects are yet to be further investigated. The identification of squalamine from the sea lamprey may offer a good model system to investigate the mechanisms of anti-angiogenesis and origin of the biological activity.

Along with squalamine, some other aminosterols with antimicrobial properties were identified from the dogfish shark (Rao et al., 2000). One of them was an aminosterol called MSI 4316, with sperimine of squalamine substituted with spermine. This compound was found to have appetite suppressant and anti-diabetic properties (Zasloff et al., 2001). This finding indicates that aminosterols may have some hormonal functions that involves in lipid and glucose metabolisms and regulation of the central nervous system. Although it is not clear yet whether this MSI 4316 is present in the sea lamprey, considering their unique life history involving feeding and fasting stages, it is possible that the sea lamprey can produce this compound and use it as a hormone that controls their feeding behavior throughout their complex life history.

Since its initial isolation from the shark, squalamine has attracted a lot of attention because of its potential as a cancer therapeutic agent. But little is known about its biosynthetic pathways and the mechanisms that it exert it effects in the organism where it was originated from. In the previous study, it was suggested that squalamine could be synthesized in the liver because the liver is well known for a site of bile acid biosynthesis (Moore et al., 1993). However, whether synthesis of squalamine completes in the liver or the spermidine conjugation occurs at some other tissues remains to be further investigated.

Furthermore, comparative studies of sqalamine in the vertebrate can offer insight into the evolution of antimicrobial factors as part of innate immune system.

To summarize, we have isolated an aminosterol, squalamine, from the blood cells of the sea lamprey. This finding suggests that this antimicrobial and anti-angiogenic compound could be more widely distributed throughout the vertebrates because it is believed to be involved in innate immunity. More studies are required to understand its biosynthetic pathways and action mechanisms.

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Figure legend

Fig. 1. Cation exhchange chromatography of solid phase extract from the sea lamprey blood cells. 50 % acetonitrile/0.1% trifluoroacetic acid eluate of C18 Sep-Pak was loaded on to a HiPrep SF column and eluted with 0-1M of NaCl over 60 min. 5 min factions were collected and desalted using Sep-Paks and antimicrobial activity was assayed. Positive fractions are indicated by dashed line.

Fig. 2. Gel filtration chromatography of pooled fractions from the cation exchange chromatography. Isocratic elution was performed with 30 % acetonitrile/0.1 % trifluoroacetic acid for 60 min. 2 min fractions were collected. Fractions eluting 23-26 min showed strong antimicrobial activity, as indicated by dashed line.

Fig. 3. Reversed phase high pressure liquid chromatography of the pooled fractions from the gel filtration chromatography. Pooled fractions were loaded on to a C18 Jupiter column and eluted with a linear gradient of 20-50 % acetonitrile/0.1% trifluoroacetic acid for 50 min. Most antimicrobial activity was found in the fractions eluting at 47-50 min (dashed line).

Fig. 4. FAB-MS/MS analysis of antimicrobial factor from the sea lamprey blood cells. An aliquot of the pooled fractions showing strong antimicrobial activity was subjected to FAB-MS and FAB-MS/MS. Major daughter ions were found at m/z 548 and at m/z 530, indicating fragmentation of SO_3 (-80) and additional water (-

18). Database search revealed that the antimicrobial substance found in the sea lamprey is squalamine.

Fig. 5. Chemical structure of squalamine. Sqaualamine is a spermidine conjugate of a sulfated bile acid with 5α configuration. This antimicrobial compound shares some chemical structures with previously know sea lamprey bile acids.

Fig. 6. Confirmation of squalamine as the antimicrobial factor in the sea lamprey. Both squalamine lactate and an aliquot of the pooled active fractions were derivatized using o-phthaldialdyhyde and separated on a C18 column. Please note that squalamine lactate (A) and an aliquot of active fractions (B) elute at the same time in the separate HPLC runs. Further, coelution of squalamine lactate and an aliquot of active fractions is observed (C), indicating the chromatographic properties of both compounds are identical.

Fig 1

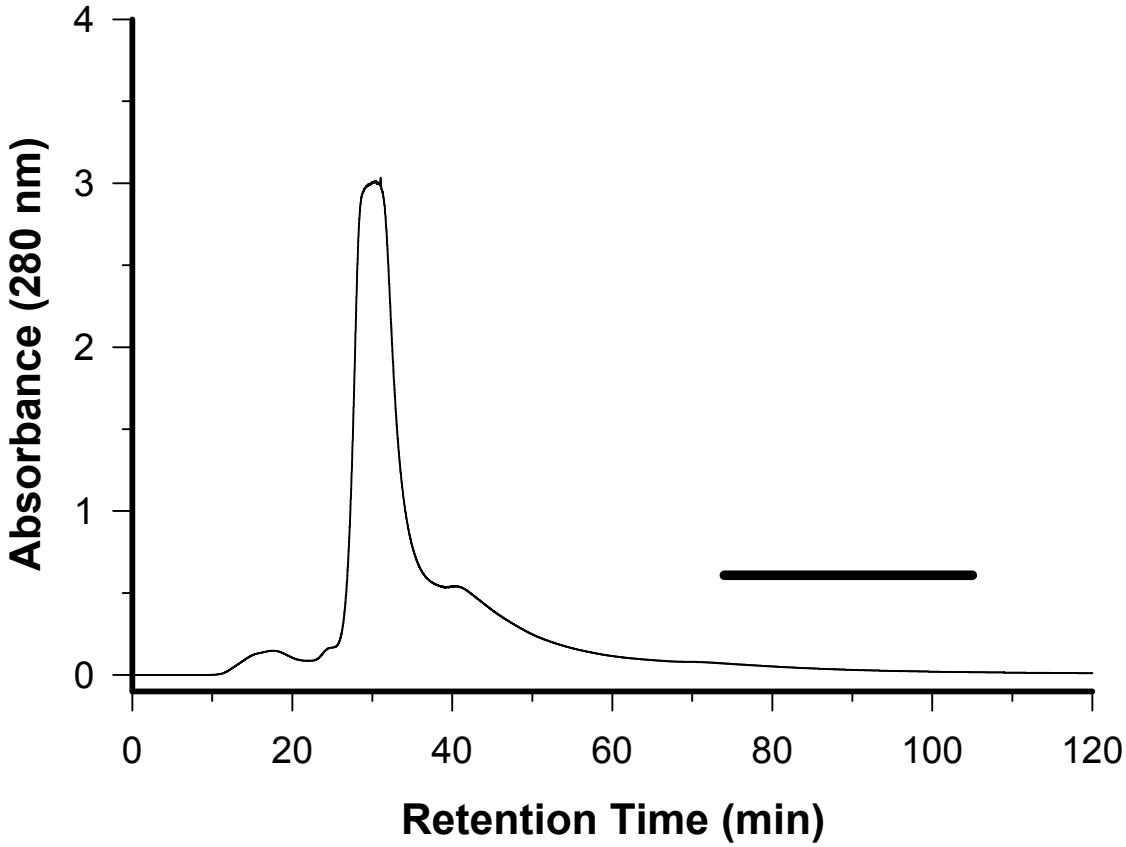
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Fig 2

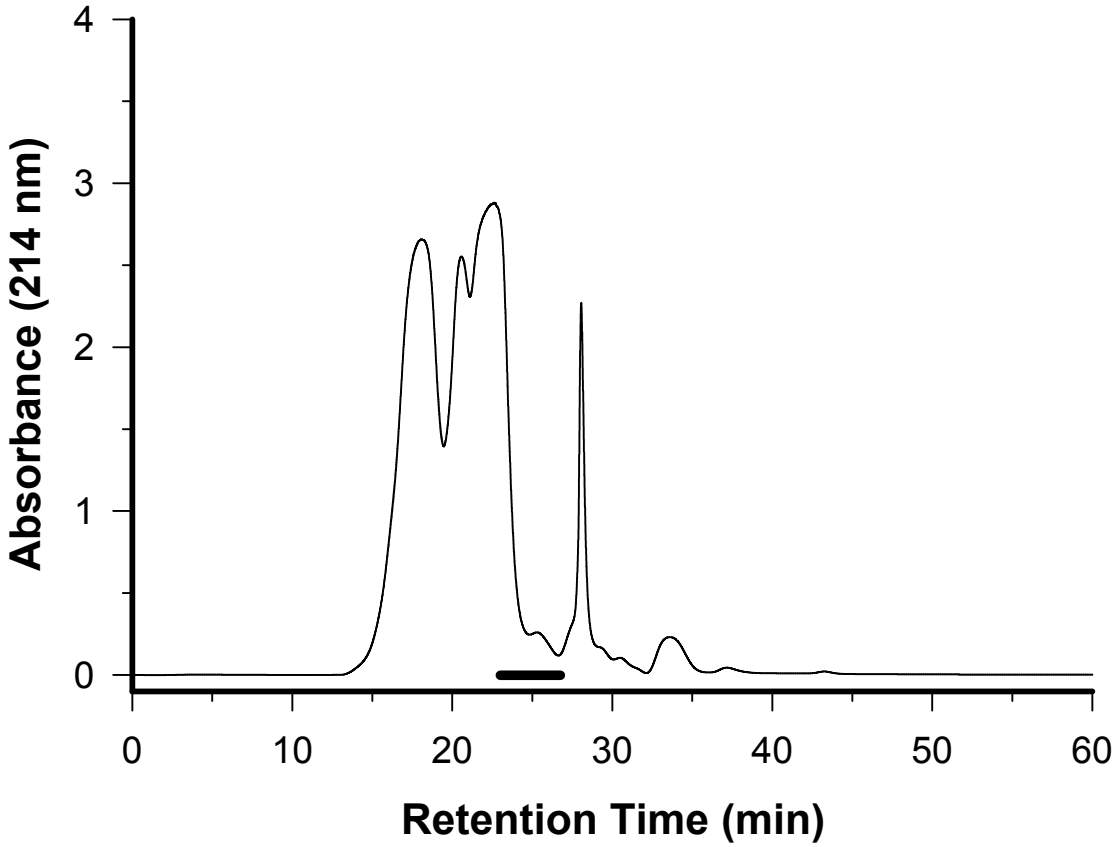

Fig 3

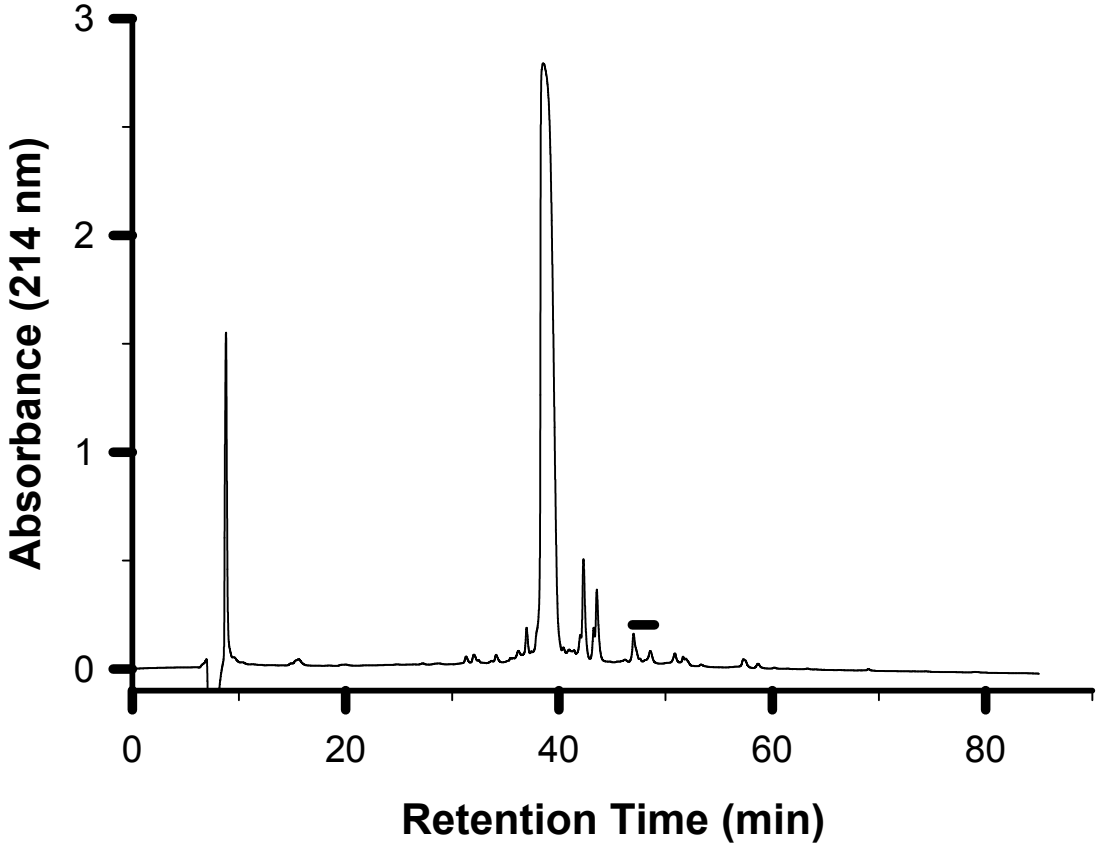

Fig 4

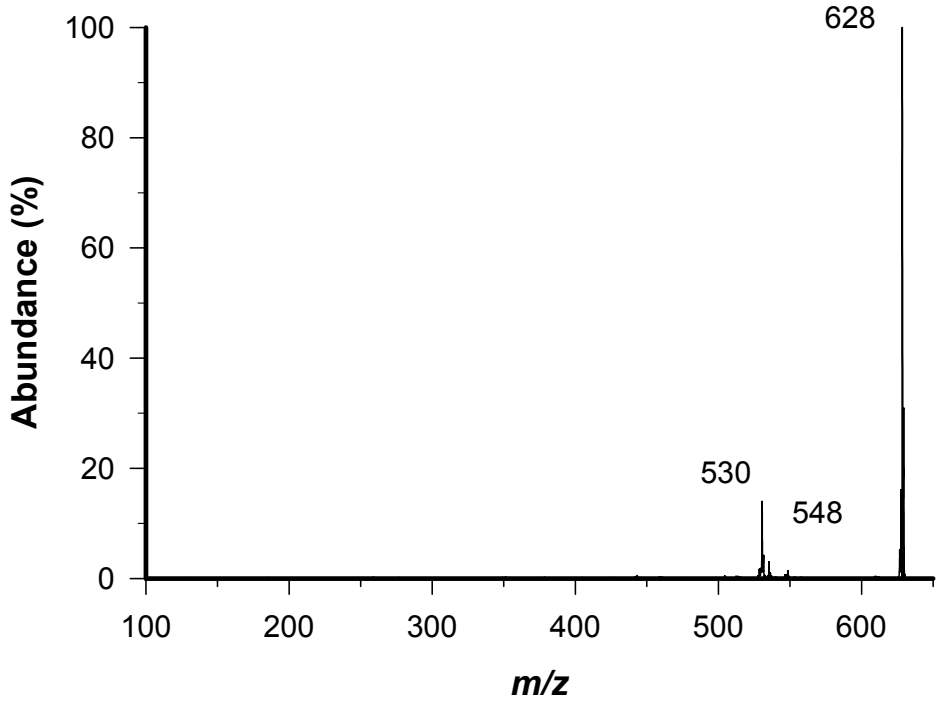
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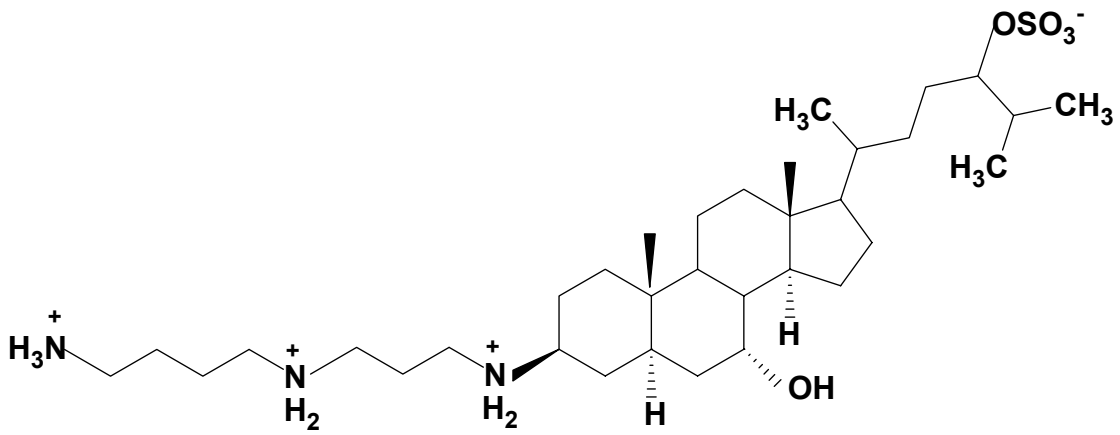
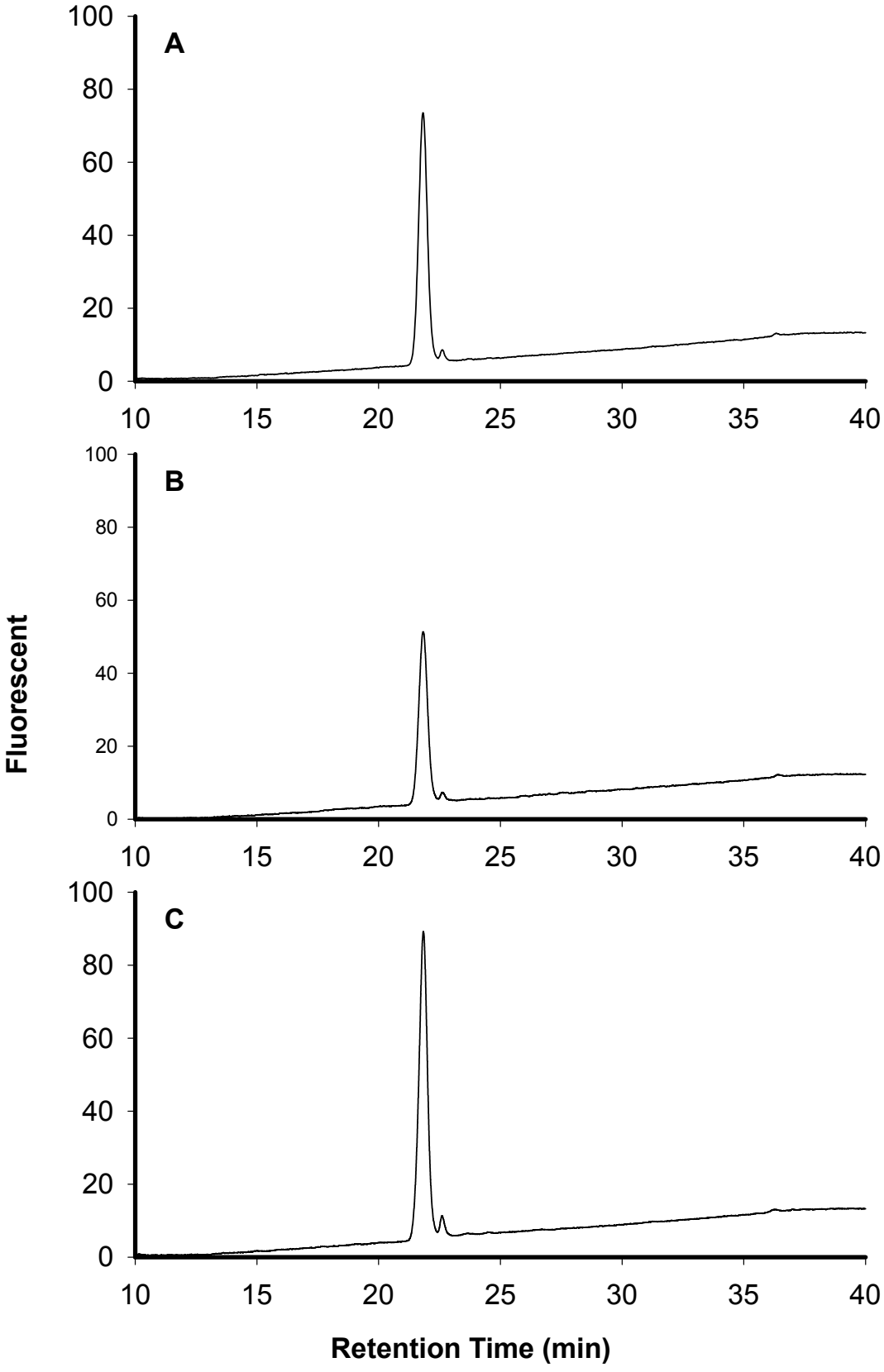
Fig 5

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60
40
20
0
10
15
20
25
30
35
40
Fluorescent
Retention Time (min)