Enzymatic conversion of Cypridina luciferyl sulfate to Cypridina luciferin with coenzyme A as a sulfate acceptor in *Cypridina* (*Vargula*) *hilgendorfii*

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Abbreviations: 3′-AMP, adenosine 3′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; APS, adenosine-5′-phosphosulfate; CoA, coenzyme A; 5′-CMP, cytidine 5′-monophosphate; 5′-dAMP, 2′-deoxyadenosine-5′-monophosphate; 5′-GMP, guanosine 5′-monophosphate; 5′-IMP, inosine 5′-monophosphate; PAP, 3′-phosphoadenosine-5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; 5′-UMP, uridine 5′-monophosphate; 5′-XMP, xanthosine 5′-monophosphate; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid; LC/ESI-TOF-MS, liquid chromatography equipped with electrospray ionization time-of-flight mass spectrometry; $I_{max,}$ maximum luminescence intensity, RLU, relative light units.

ABSTRACT

In the luminous ostracod *Cypridina* (presently *Vargula*) *hilgendorfii,* Cypridina luciferyl sulfate (3-enol sulfate of Cypridina luciferin) is converted to Cypridina luciferin by a sulfotransferase with 3′-phosphoadenosine-5′-phosphate (PAP) as a sulfate acceptor. The resultant Cypridina luciferin is used for the luciferase–luciferin reaction of *Cypridina* to emit blue light. The luminescence stimulation with major organic cofactors was examined using the crude extracts of *Cypridina* specimens, and we found that the addition of coenzyme A (CoA) to the crude extracts significantly stimulated luminescence intensity. Further, the light-emitting source in the crude extracts stimulated with CoA was identified as Cypridina luciferyl sulfate, and we demonstrated that CoA could act as a sulfate acceptor from Cypridina luciferyl sulfate. In addition, the sulfate group of Cypridina luciferyl sulfate was also transferred to adenosine 5′-monophosphate (5′-AMP) and adenosine 3′-monophosphate (3′-AMP) by a sulfotransferase. The sulfated products corresponding to CoA, 5′-AMP, and 3′-AMP were identified using mass spectrometry. This is the first report that CoA can act as a sulfate acceptor in a sulfotransferase reaction.

INTRODUCTION

The bioluminescence system of the sea firefly, *Cypridina* (presently *Vargula*) *hilgendorfii*, is a simple enzymatic reaction of a luciferin (Cypridina luciferin) and a luciferase (Cypridina luciferase) with molecular oxygen (O_2) [1-3]. Cypridina luciferin (**1**) [4-6] is classified as an imidazopyrazinone-type luciferin, and Cypridina luciferase (61 kDa protein) [7, 8] catalyzes the oxidation of Cypridina luciferin to produce blue light (λ_{max} = 460 nm), oxyluciferin (**2**) and CO_2, (**Fig. 1A**). Another well-known imidazopyrazinone-type luciferin is coelenterazine (**3**) [9], which is widely used as a light-emitting source for various luciferases and photoproteins from marine organisms such as *Renilla, Oplophorus, Gaussia, Periphylla, and Aequorea* [10]. It has been reported that Cypridina luciferin and coelenterazine are biosynthesized from free L-amino acids in living specimens of *Cypridina* and *Metridia*, respectively [11-14]. However, no experimental evidence of the biosynthetic pathway for these luciferins, including their intermediates, has been reported to date.

<Figure 1>

During studies on the biosynthesis of Cypridina luciferin in *C. hilgendorfii* [11-13], we identified a new sulfate derivative of Cypridina luciferin using mass spectrometry and named it "Cypridina luciferyl sulfate" (Cypridina luciferin 3-enol sulfate) (**4**) [15]. Cypridina luciferyl sulfate was found to be more stable than Cypridina luciferin in aqueous solutions and was proposed to be the stored form of Cypridina luciferin in *Cypridina* specimens [15]. Furthermore, we demonstrated that Cypridina luciferyl sulfate could be converted to Cypridina luciferin, presumably through an intermediate of 3-enol form of Cypridina luciferin (**5**) as a tautomeric form of Cypridina luciferin, in the presence of 3′-phosphoadenosine-5′-phosphate (PAP) (**6**) as a sulfate acceptor, by incubation with crude extracts of *Cypridina* specimens (**Fig. 1A**). In addition, the conversion of Cypridina luciferin to Cypridina luciferyl sulfate was confirmed in the presence of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (**7**) as a sulfate donor. Thus, the process of sulfation of Cypridina luciferin is a reversible enzymatic reaction, catalyzed by a sulfotransferase enzyme in *Cypridina* specimens [15].

Cypridina luciferyl sulfate has a core structure identical to that of coelenterazine 3-enol sulfate (**8**), which was identified in the sea pansy, *R. reniformis* (**Fig. 1B**) [16, 17]. The sulfation of coelenterazine to coelenterazine 3-enol sulfate in *Renilla* was shown to

be catalyzed by a sulfotransferase, but no such enzyme was isolated [18]. Another sulfate derivative of coelenterazine is coelenterazine disulfate (**9**), which is known as a luciferin from the firefly squid, *Watasenia scintillans* [19]. Presumably, coelenterazine disulfate might be produced by a phenol sulfotransferase.

In the course of investigating for the biosynthetic pathway of Cypridina luciferin, we tentatively examined the effects of organic cofactors including NADH, NAD^+, FMN, FAD, ATP, and coenzyme A (CoA) on the luminescence intensity using crude extracts of *Cypridina* specimens. Of these cofactors, we found that the addition of CoA to the crude extracts significantly stimulated the luminescence intensity. Based on the luminescence stimulation with CoA as an indicator, we attempted to isolate a light-emitting source from crude extracts of *Cypridina* specimens.

In this study, we describe that Cypridina luciferyl sulfate, which was identified by us in 2014 [15], is the light-emitting source stimulated with CoA in crude extracts of *Cypridina* specimens and that CoA can act as a sulfate acceptor from Cypridina luciferyl sulfate to produce Cypridina luciferin on the micromolar order. Furthermore, we demonstrate that the other adenosine derivatives of adenosine 5′-monophosphate (5′-AMP) and adenosine 3′-monophosphate (3′-AMP) can also act as efficient sulfate acceptors on the millimolar order from Cypridina luciferyl sulfate by a sulfotransferase and they stimulate light emission in crude extracts of *Cypridina* specimens. We confirmed the sulfated products of CoA, 5′-AMP, and 3′-AMP using LC/ESI-TOF-MS analyses.

MATERIALS AND METHODS

***Chemicals*:** Adenosine, adenosine 3′, 5′-diphosphate (3′-phosphoadenosine-5′-phosphate: PAP), adenosine 5′-monophosphate (5′-AMP), and uridine 5′-monophosphate disodium salt (5′-UMP) were obtained from Kanto Chemical Co. (Tokyo, Japan), Sigma (St. Louis, MO, USA), Oriental Yeast Co. (Tokyo, Japan), and Combi-Blocks (San Diego, CA, USA), respectively. Cytidine 5′-monophosphate disodium salt (5′-CMP), 2′-deoxyadenosine 5′-monophosphate (5′-dAMP) and guanosine 5′-monophosphate disodium salt (5′-GMP) were obtained from Chem-Impex International (Wood Dale, IL, USA). Xanthosine 5′-monophosphate sodium salt (5′-XMP) and adenosine 5′-phosphosulfate (APS) were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). FAD and adenosine 3′-monophosphate (3′-AMP) were obtained from Tokyo

Chemical Industry Co. (Tokyo, Japan). NAD^+, NADH, FMN, and inosine 5′-monophosphate disodium salt (5′-IMP) were obtained from Nacalai Tesque, Inc (Kyoto, Japan). Coenzyme A (CoA) was purchased from Wako Pure Chemical Industries and Oriental Yeast Co. All other chemical reagents were obtained from Wako Pure Chemical Industries and Nacalai Tesque.

***Preparation of crude extracts of Cypridina specimens*:** Specimens of *C. hilgendorfii* were collected at Chita (Aichi, Japan) on August 4, 2011, and Naruto (Tokushima, Japan) on August 24, 2017. The live specimens were quickly frozen in liquid nitrogen or dry ice and stored at −80 °C until use. The frozen *Cypridina* specimens were homogenized in an appropriate buffer using a plastic pestle in a 1.5-mL tube in an ice-bath. The homogenates were centrifuged at 20,600 × *g* for 20 min at 4 °C using a model 3500 centrifuge (Kubota Co., Tokyo, Japan) and the resultant supernatant was used as "crude extracts" of *Cypridina* specimens in this manuscript.

***Preparation of "G25-fraction" from crude extracts of Cypridina specimens for luminescence assay*:** The crude extracts of *Cypridina* specimens were prepared from the frozen specimens (102.7 mg, wet weight) in 300 μL of 100 mM glycine-NaOH (pH 7.5) and were kept on ice. To remove low-molecular weight compounds such as nucleotides and cofactors, the crude extracts were subjected to gel filtration using a Sephadex G-25F column (ø6 mm × 88 mm; GE Healthcare Life Sciences) at room temperature. The void volume fractions (1 mL) containing Cypridina luciferase and sulfotransferase(s) were collected and concentrated to 100 μL using a Millipore-Amicon ultra-0.5 mL centrifugal unit (MWCO 10,000). The concentrated fraction is referred to as the "G25-fraction" in this manuscript. During the course of preparation, Cypridina luciferin in the G25-fraction was consumed by endogenous Cypridina luciferase, and the G25-fraction did not contain Cypridina luciferin and cofactors. Thus, the G25-fraction could be used as enzyme sources of Cypridina luciferase and sulfotransferase(s) for luminescence assays.

***Preparation of highly purified coenzyme A (CoA)*:** CoA (2.1 mg, Lot No. 132605; Oriental Yeast Co.) was purified using reversed-phase HPLC (RP-HPLC). RP-HPLC was performed with an ODS column (Cosmosil 5C18-AR, ø20 × 250 mm; Nacalai Tesque) using two CCPS pumps (Tosoh Co., Tokyo, Japan) and a UV-8020 detector (Tosoh Co.)

under the following conditions: mobile phase, 10% CH_3OH in H_2O containing 0.01% TFA; flow rate, 5.0 mL/min; and detection, 280 nm. CoA was eluted at 27.4 min, and its yield of CoA was 2.0 mg.

***Identification of Cypridina luciferyl sulfate as a light-emitting source stimulated with CoA*:** To isolate the light-emitting compound stimulated with CoA, the crude extracts (900 μL) from *Cypridina* specimens (wet weight, 562.1 mg) were applied on an ODS (Wakogel (R) 100C18) column (ø8 × 122 mm) and eluted in a stepwise manner with 18 mL of methanol/H_2O (1:9, 4:6, and 1:0). The major fraction stimulated with CoA was eluted in 100% methanol, evaporated and dissolved in 20 μL of methanol. The methanol fraction was further purified by RP-HPLC with an ODS column, Cosmosil protein-R (ø4.6 × 150 mm; Nacalai Tesque), under the following conditions: mobile phase, CH_3OH/H_2O increased from 50% to 70% (v/v) in 20 min; flow rate, 0.8 mL/min; and detection, 280 nm. The isolated fraction at 8.5 min was subjected to LC/ESI-TOF-MS and the light-emitting source stimulated with CoA was identified as Cypridina luciferyl sulfate [15]. The yield was 23 μg, which was estimated by calculating the peak area in the HPLC analysis at 280 nm, as previously described [15]. The purified Cypridina luciferyl sulfate was used for the assays.

***Determination of luminescence activity*:** The luminescence activity of Cypridina luciferase was determined using an Atto (Tokyo, Japan) AB2270 luminometer (Ver.1.025) with an F0 filter (in the absence of cut filters, F1 [HOYA, O56] and F2 [HOYA, R62]) for 60 s in 0.1-s intervals in a glass tube (Nippon Electric Glass Co., Shiga, Japan). The maximum luminescence intensity (I_{max}) was determined and is shown as relative light units (RLU). In our assay conditions using an AB2270 luminometer, 1 RLU was estimated to be 1.2×10^3 photons/0.1 s, based on the I_{max} value of recombinant aequorin (JNC Corp., Japan) as a light standard, as previously described [20].

***Stimulation of luminescence intensity by addition of cofactors to crude extracts of Cypridina specimens*:** The crude extracts of *Cypridina* specimens were prepared from frozen specimens (3 bodies). The frozen specimens were homogenized in 100 μL of 100 mM glycine-NaOH (pH 8.5) and were then allowed to stand for 6 h on ice. After endogenous luminescence in the crude extracts had disappeared, the luminescence

intensity was determined by adding 1 μL of 100 mM cofactors (NADH, NAD^+, FMN, FAD, ATP-300 mM $MgCl_2$, and CoA) dissolved in 100 mM glycine-NaOH (pH 8.5) to 100 μL of 100 mM glycine-NaOH (pH 8.5) containing 5 μL of crude extracts of *Cypridina* specimens.

***Stimulation of luminescence intensity by addition of CoA to crude extracts of Cypridina specimens*:** The luminescence reaction was started by adding 1 μL of 100 mM CoA dissolved in 100 mM glycine-NaOH (pH 7.5) to 100 μL of 100 mM glycine-NaOH (pH 7.5) containing 5 μL of crude extracts of *Cypridina* specimens. The luminescence activity was determined using a luminometer for 60 s and the mean value of I_{max} (n = 3) was calculated.

***Stimulation of luminescence activity by adding various concentrations of PAP and nucleotide derivatives to the mixture of G25-fraction of Cypridina specimens and purified Cypridina luciferyl sulfate*:** The reaction mixture contained 5 μL of the G25-fraction from crude extracts of *Cypridina* specimens and the purified Cypridina luciferyl sulfate (320 ng) in 100 μL of 100 mM glycine-NaOH (pH 7.5). The stimulation of luminescence activity was examined by adding various concentrations of PAP and nucleotide derivatives. The luminescence activity was determined for 60 s using a luminometer and the mean value of I_{max} (n = 3) was calculated.

***Inhibition of luminescence activity by addition of nucleotide derivatives to G25-fraction of Cypridina specimens*:** The luminescence reaction was started by adding 5 μL of the G25-fraction to 100 μL of 100 mM glycine-NaOH (pH 7.5) containing Cypridina luciferyl sulfate (320 ng) and 1 μL of 10 mM nucleotide derivatives. After determining luminescence activity for 60 s, 1 μL of 0.1 mM PAP was added to the reaction mixture and the luminescence activity was determined further for 60 s. The mean value of I_{max} (n = 3) was then calculated.

***Mass spectrometry*:** To identify the reaction product of adenosine derivatives using crude extracts of *Cypridina* specimens, 1 μL of 1 mM 5′-AMP, 3′-AMP, or CoA was added to 100 μL of 100 mM glycine-NaOH (pH 7.5) containing Cypridina luciferyl sulfate (1.7 μg) and 5 μL of the G25-fraction and incubated for 15 min at room temperature (20 ~

25 °C). The reaction mixture was centrifuged at 20,600 × *g* for 20 min at 4 °C, and the supernatant was analyzed using LC/ESI-TOF-MS. Mass spectra were acquired using a XEVO Q-TOF MS System (Waters Japan, Tokyo, Japan) coupled with an on-line ACQUITY UPLC® System (Waters Japan). The ESI capillary voltage was set at 2.9 kV in negative ion mode. The source and desolvation temperature were set at 120 °C and 400 °C, respectively. The desolvation and cone gas flows were 800 L/h and 50 L/h, respectively. The sample and extraction cone voltage were set at 40 V and 2.0 V, respectively. The collision energy was set at 6 eV to collect sample precursor (MS) data and at 30 eV to obtain sample fragmentation (MS/MS) data. An ODS column, Cosmosil AR-II (ø2.0 × 150 mm; Nacalai Tesque), was used under the following conditions: mobile phase, CH_3CN/H_2O increased from 0% to 10% (v/v) in 10 min; flow rate, 0.2 mL/min. Here, 5′-AMP with *m/z* 346, adenosine 5′-phosphosulfate (APS) with *m/z* 426, 3′-AMP with *m/z* 346, and adenosine-3′-phosophosulfate with *m/z* 426 were eluted at 2.0, 9.2, 2.5, and 10.0 min, respectively.

The sulfated adenosine and sulfated CoA were analyzed by the infusion method for MS analysis using the XEVO Q-TOF MS System. APS; HRMS(ESI): *m/z* calculated for $C_{10}H_{14}N_5O_{10}PS$ $[M-H]^-$ 426.0121, was found to be 426.0118. Adenosine-3′-phosphosulfate; HRMS(ESI): *m/z* calculated for $C_{10}H_{14}N_5O_{10}PS$ $[M-H]^-$ 426.0121, was found to be 426.0127. CoA-sulfate; HRMS(ESI): *m/z* calculated for $C_{21}H_{36}N_7O_{19}P_3S_2$ $[M-H]^-$ 846.0642, was found to be 846.0652.

RESULTS AND DISCUSSION

Effect of the addition of cofactors on the luminescence intensity of crude extracts of *Cypridina* specimens

In the biosynthetic pathway of Cypridina luciferin in *Cypridina* specimens, we hypothesized that some cofactors such as NADH, NAD^+, FMN, FAD, ATP, and CoA may likely contribute to the biosynthetic pathway of Cypridina luciferin from the three L-amino acids of tryptophan, isoleucine, and arginine. To investigate the effects of cofactors on this pathway, we examined the stimulation of luminescence by cofactors using crude extracts of *Cypridina* specimens. The crude extracts prepared in 100 mM glycine-NaOH (pH 8.5) were allowed to stand in an ice-bath for 6 h. After disappearance of endogenous

243 luminescence in the crude extracts, the stimulation of luminescence was determined by
244 adding cofactors at a final concentration of 1 mM NADH, NAD^+, FMN, FAD, ATP, and
245 CoA. The results revealed that CoA significantly stimulated the luminescence activity,
246 whereas ATP, NADH, and NAD^+ weakly stimulated the luminescence activity (**Fig. 2**).

247 <Figure 2>

248 To confirm the luminescence stimulation with CoA, CoA from a commercial source
249 (Oriental Yeast Co.) was further purified by HPLC and used for the stimulation
250 experiment (**Supplementary Fig. S1**). Thus, CoA is an effective cofactor to stimulate the
251 luminescence activity of crude extracts in *Cypridina* specimens.
252
253 **Optimal pH for stimulating luminescence activity of crude extracts and the G25-**
254 **fraction**
255 The optimal pH for luminescence stimulation of crude extracts with CoA was found
256 to be 8.0 ~ 8.5 in 100 mM glycine-NaOH (**Supplementary Fig. S2**). The luminescence
257 intensity in 100 mM glycine-NaOH at pH 8.0 ~ 8.5 was over 3-fold higher than that in
258 100 mM Tris-HCl at pH 8.0 ~ 8.5. Therefore, 100 mM glycine-NaOH was chosen as the
259 homogenizing solution for *Cypridina* specimens, although the reason for the higher
260 luminescence activity in glycine-NaOH was not clarified. In contrast, the luminescence
261 stimulation of the G25-fraction with PAP or CoA at pH 7.5 was approximately 2-fold
262 higher than that at pH 8.5 (**Supplementary Fig. S3**). From these results, the assay with
263 the G25-fraction was performed in 100 mM glycine-NaOH at pH 7.5.
264
265 **Identification of 3-enol sulfate of Cypridina luciferin as a light-emitting source by**
266 **stimulating with CoA**
267 To isolate the compound acting as a light-emitting source stimulated with CoA, the
268 crude extracts of *Cypridina* specimens were applied on an ODS column and each fraction
269 was monitored by the luminescence stimulation with CoA using crude extracts. A
270 compound acting as a light-emitting source was found in the methanol fraction and was
271 further separated under neutral pH conditions by RP-HPLC using an ODS column, and
272 was then analyzed by LC/ESI-TOF-MS. Unexpectedly, mass spectrometry data of the
273 isolated compound was identical to that of Cypridina luciferyl sulfate, which we had
274 isolated previously [15]. Thus, we concluded that the light-emitting source stimulated

with CoA in crude extracts of *Cypridina* specimens is Cypridina luciferyl sulfate.

From these results, the luminescence stimulation with CoA was explained by the formation of Cypridina luciferin from Cypridina luciferyl sulfate and CoA, and Cypridina luciferin formed was used for the luciferin–luciferase reaction (**Fig. 1A**). The formation of Cypridina luciferin might be catalyzed by a sulfotransferase, and CoA may act as a sulfate acceptor. However, it has not been reported that CoA acts as a sulfate acceptor in the general sulfotransferase reaction [21-23].

Stimulation of the luminescence activity in the G25-fraction of *Cypridina* specimens by addition of CoA, 5′-AMP, and 3′-AMP

CoA is a well-known cofactor that is one of adenosine derivatives, and the effects of other nucleotide derivatives (**Fig. 3 and Supplementary Fig. S4**) on the luminescence stimulation in crude extracts of *Cypridina* specimens were further examined.

<Figure 3>

As we reported [15], the crude extracts contained Cypridina luciferase and sulfotransferase(s). The crude extracts were applied on Sephadex G25 column to remove sulfate derivatives and endogenous nucleotide derivatives such as PAP and PAPS from crude extracts, and the void volume fractions were used as the "G25-fraction" containing Cypridina luciferase and sulfotransferase(s). For determining the stimulation with cofactors, the G25-fraction and purified Cypridina luciferyl sulfate were used in assays. The effects of nucleotide derivatives at various concentrations on luminescence stimulation were summarized in **Fig. 4A**.

<Figure 4>

The luminescence activity following the addition of PAP to the G25-fraction containing purified Cypridina luciferyl sulfate was significantly stimulated at 10^{-8} to 10^{-6} M. This is a general property of sulfotransferases with PAP, which is a similar reaction to the formation of coelenterazine from coelenterazine 3-enol sulfate with PAP in *Renilla* specimens [18]. Thus, Cypridina luciferyl sulfate with PAP was converted to Cypridina luciferin, followed by catalyzing the luminescence reaction with Cypridina luciferase in the G25-fraction [15] (**Fig. 1**). Interestingly, the adenine derivatives of 5′-AMP and 3′-AMP also showed significant stimulation at concentrations of more than 10^{-4} M. The stimulation intensity at 10^{-3} M was in the following order (relative intensity to 5′-

AMP, %): 5′-AMP (100) > 3′-AMP (57) > CoA (39). Although CoA showed less effect on the luminescence intensity, the effective molar level was 10^{-6} M, which is close to the effective concentration of PAP (**Fig. 4A**). The luminescence patterns stimulated with PAP, CoA, 5′-AMP, and 3′-AMP showed the steady-stay kinetics (**Fig. 5**).

<Figure 5>

Other nucleotide derivatives including adenosine, 5′-CMP, 5′-GMP, 5′-IMP, 5′-UMP, and 5′-XMP did not stimulate the luminescence activity. These results suggested that the adenine moiety of cofactors was essential for *Cypridina* sulfotransferase(s) as the sulfate acceptor from Cypridina luciferyl sulfate. Thus, sulfotransferase(s) in *Cypridina* specimens could efficiently use CoA, 5′-AMP, and 3′-AMP as a sulfate acceptor from Cypridina luciferyl sulfate and produced Cypridina luciferin, similar to the case with PAP. However, the effective concentrations of 5′-AMP and 3′-AMP at 10^{-3} M were high and they might not be use as sulfate acceptors in living *Cypridina* specimens. Presumably, CoA is a potential candidate for the sulfate acceptor in Cypridina sulfotransferase(s) *in vivo*, similar to the case of PAP.

It has been reported that CoA acts as a competitive inhibitor for bovine phenol sulfotransferase (bSUTA1) with PAPS [24-27]. As the core structure of adenosine-3′-phospho-5′-phosphate moiety in CoA is identical to that of PAPS and PAP, they could bind the catalytic site in human phenol sulfotransferase (SULTA1, PDB code 1LS6) [28]. Using the G25-fraction of *Cypridina* specimens, we examined the inhibitory effects of various concentrations of CoA on luminescence activity in the presence of PAP (1 μM, 10^{-6} M). As shown in **Fig. 4B**, CoA (10^{-6} to10^{-3} M) showed the moderate inhibition on luminescence activity. Furthermore, the inhibition with various nucleotide derivatives (0.1 mM) in the presence of PAP (0.1 μM) were also examined, and only CoA showed strong inhibition of luminescence activity (**Fig. 4C**).

From these results, we consider that CoA may be the competitive inhibitor against PAP in Cypridina sulfotransferase(s) and can also act as a sulfate acceptor. This is the first report that a sulfotransferase could use CoA as a sulfate acceptor among known sulfotransferases, and the sulfation of CoA was confirmed using LC/ESI-TOF-MS, as described in the following text.

Mass spectral analyses of the sulfated products of 5′-AMP, 3′-AMP and CoA

The sulfated products of 5′-AMP, 3′-AMP and CoA, which acted as sulfate acceptors from Cypridina luciferyl sulfate in the sulfotransferase reaction, were confirmed using LC/ESI-TOF-MS [29].

<Figure 6>

i) 5′-AMP as a sulfate acceptor:

The mass spectrum of the sulfate product of 5′-AMP showed a peak of *m/z* 426.0118 ($[M-H]^-$) in negative ion mode (**Fig. 6A)**. In contrast, 5′-AMP showed a mass peak of *m/z* 346.0558 and its MS/MS spectrum showed three major product ions: *m/z* 79.0 (PO_3^-), *m/z* 97.0 ($PO_4H_2^-$) and *m/z* 134.1 ($C_5H_4N_5^-$, adenine ion) (**Fig. 7A and 7B**). This result indicated that 5′-AMP was modified with an adduct of 80 Da (SO_3). The sulfate product of *m/z* 426.0118 was fragmented to *m/z* 79.0, *m/z* 97.0, *m/z* 134.0, and *m/z* 158.9 (**Fig. 7C and 7E**). The product ion of *m/z* 158.9 corresponded to a dehydrated phosphosulfate moiety ($SO_3PO_3^-$). Thus, the sulfate product of 5′-AMP was adenosine-5'-phosphosulfate (APS) and showed the MS/MS spectrum patterns identical to that of an authentic APS **(Fig. 7D**).

<Figure 7>

ii) 3′-AMP as a sulfate acceptor:

The MS/MS spectrum of the authentic sample of 3′-AMP (*m/z* 346.0556) and its predicted fragmentation pattern are shown in **Fig. 8A and 8B**, respectively. The MS/MS spectrum of 3′-AMP showed four major product ions: *m/z* 78.9 (PO_3^-), *m/z* 97.0 ($PO_4H_2^-$), *m/z* 134.0 ($C_5H_4N_5^-$, adenine ion) and *m/z* 211.0 ($C_5H_8O_7P^-$, phosphoribose-derived ion). On the contrary, the reaction product of 3′-AMP with the crude extracts of *Cypridina* specimens showed a peak of *m/z* 426.0127 (**Fig. 6B)**, and its MS/MS spectrum showed five major product ions: *m/z* 79.0 (PO_3^-), *m/z* 97.0 ($PO_4H_2^-$ or SO_4H^-), *m/z* 134.1 ($C_5H_4N_5^-$, adenine ion), *m/z* 158.9 ($SO_3PO_3^-$), and *m/z* 211.0 ($C_5H_8O_7P^-$, phosphoribose-derived ion) (**Fig. 8C and 8D**). The signal intensity of *m/z* 211.0 in the product of sulfated 3′-AMP (**Fig. 8B**) was considerably lower than that in 3′-AMP (Fig. 6A). Thus, the sulfation of 3′-AMP was occurred at the 3′-position of adenosine, which is adenosine-3′-phosphosulfate.

<Figure 8>

iii) CoA as a sulfate acceptor:

The MS/MS spectrum of the authentic sample of CoA (*m/z* 766.1075) and its predicted fragmentation pattern are shown in **Fig. 9A and 9B** (**Supplementary Fig. S5)**, respectively. The mass spectrum of the sulfate product showed a small peak of *m/z* 846.0652 ($[M-H]^-$) in negative ion mode (**Fig. 6C)**, indicating that an adduct of CoA with 80 Da had formed. The fragment pattern in the MS/MS spectrum of *m/z* 846.0652 (**Fig. 9C and 9D, Supplementary Fig. S6**) was similar to that of CoA (**Fig. 9A and 9B, Supplementary Fig. S5**). Because the signal intensity of phosphoribose-derived ion is lower than that of phosphoribose ion, the sulfation of CoA might occur at the 3′-position of the adenosine moiety of CoA. From these results, the sulfate moiety presumably transferred to the 3′-position as phosphosulfate, which was similar to the case of 3′-AMP.

<Figure 9>

CONCLUSION

Sulfotransferase(s) in *Cypridina* specimens were able to catalyze the transfer of the sulfate group of Cypridina luciferyl sulfate to PAP, 5′-AMP, 3′-AMP and CoA as acceptors, and the resultant Cypridina luciferin was used for the luciferin–luciferase reaction. The sulfated products of CoA, 5′-AMP, and 3′-AMP were confirmed using mass spectrometry. To the best of our knowledge, this is the first report describing that CoA can act as a sulfate acceptor in a sulfotransferase reaction.

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SUPPORTING INFORMATION

Supporting Information can be found at DOI:xxxxxxxxx

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Figure legends

Fig. 1. Bioluminescence system in *Cypridina* specimens and the chemical structures of coelenterazine and its sulfate derivatives in marine organisms

A. Enzymatic conversion of Cypridina luciferyl sulfate to (*S*)-Cypridina luciferin in the presence of PAP and a sulfotransferase. The generated Cypridina luciferin is oxidized by Cypridina luciferase to produce oxyluciferin, CO_2, and light. **B**. Chemical structures of coelenterazine, coelenterazine 3-enol sulfate from *Renilla reniformis* (sea pansy), and coelenterazine disulfate (*Watasenia* luciferin) from *Watasenia scintillans* (firefly squid).

Fig. 2. Stimulation of luminescence activity in soluble fraction of *Cypridina* specimens by addition of various cofactors

The assay conditions are described in Materials and methods.

Fig. 3. Chemical structures of PAP and nucleotide derivatives

Fig. 4. Effects of various nucleotide derivatives on the luminescence activity of the G25-fraction prepared from crude extracts of *Cypridina* specimens

A. Stimulation of luminescence activity of the G25-fraction with PAP, CoA, 5′-AMP, and 3′-AMP at various concentrations. **B**. Inhibitory effect of CoA on luminescence activity stimulated with PAP. Closed squares indicate luminescence activity following the addition of CoA to the reaction mixture containing the G25-fraction and Cypridina luciferyl sulfate. Closed circles indicate the luminescence activity stimulated by the addition of PAP (at a final concentration of 10^{-6} M) to the reaction mixture containing various concentrations of CoA. **C**. Effects of nucleotide derivatives on luminescence activity of the G25-fraction in the presence of PAP. Open bars indicate luminescence activity following the addition of nucleotide derivatives (at a final concentration of 10^{-4} M) to the reaction mixture containing the G25-fraction and Cypridina luciferyl sulfate. Closed bars indicate luminescence activity following the addition of PAP (at a final concentration of 10^{-6} M) to the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and nucleotide derivatives (at a final concentration of 10^{-4} M).

Fig. 5. Stimulation of luminescence activity by adding PAP, CoA, 5′-AMP, and 3′-AMP into the G25-fraction of *Cypridina* specimens

A. PAP at 10^{-7} M and 10^{-8} M. **B**. CoA at 10^{-5} M and 10^{-6} M. **C**. 5′-AMP at 10^{-3} M and 10^{-4} M. **D**. 3′-AMP at 10^{-3}M and 10^{-4} M. The reaction mixture contains Cypridina luciferyl sulfate (320 ng) in 100 μL of 100 mM glycine-NaOH (pH 7.5). The luminescence reaction was started by adding 5 μL of the G25-fraction of *Cypridina* specimens and measured for 60 s, followed by adding 1 μL of PAP (1 μM and 10 μM), CoA (0.1 mM and 1 mM), 5′-AMP (10 mM and 100 mM), and 3′-AMP (10 mM and 100 mM), respectively, and luminescence activity was further measured. Arrow indicates the point when adenosine derivative was added to the reaction mixture.

Fig. 6. Mass spectra of the reaction products from 5'-AMP, 3'-AMP and CoA by incubation of Cypridina luciferyl sulfate and the G-25 fraction

A. MS spectrum of the reaction product from 5'-AMP in negative-ion mode. **B**. MS spectrum of the reaction product from 3'-AMP in negative-ion mode. **C**. MS spectrum of the reaction product from CoA in negative-ion mode.

Fig. 7. Negative-ion mode fragmentation of 5′-AMP and the reaction product (*m/z* 426) from 5′-AMP

A. MS/MS spectrum of 5′-AMP. **B**. Predicted mass fragmentation of 5′-AMP. **C**. MS/MS spectrum of *m/z* 426 from the reaction product with 5′-AMP, Cypridina luciferyl sulfate, and the G-25 fraction. **D**. MS/MS spectrum of APS. **E**. Predicted mass fragmentation of APS.

Fig. 8. Negative-ion mode fragmentation of 3′-AMP and the reaction product (*m/z* 426) from 3′-AMP

A. MS/MS spectrum of 3′-AMP. **B**. Predicted mass fragmentation of 3′-AMP. **C**. MS/MS spectrum of *m/z* 426 from the reaction product with 3′-AMP, Cypridina luciferyl sulfate, and the G-25 fraction. **D**. Predicted structure of *m/z* 426 in **C**, and predicted fragmentation of adenosine 3′-phosphosulfate.

Fig. 9. Negative-ion mode fragmentation of CoA and the sulfated product (*m/z* 846) of CoA

551 **A.** MS/MS spectrum of CoA. **B.** Predicted mass fragmentation of CoA. **C.** MS/MS
552 spectrum of *m/z* 846 from the reaction product with CoA, Cypridina luciferyl sulfate and
553 the G25-fraction. **D**. Predicted structure of *m/z* 846 for CoA-sulfate in **C**, and predicted
554 fragmentation of CoA-sulfate.
555

SUPPORTING INFORMATION

Fig. S1. Purification of CoA from the commercial source and CoA stimulation of luminescence activity in crude extracts of *Cypridina* specimens

A. CoA (Wako Pure Chemical Industries, Lot No. KWK2017). **B**. CoA (Oriental Yeast Co., Lot No. 132605). **C**. Purified CoA by HPLC from CoA (Oriental Yeast Co., Lot No. 132605). The reaction mixture contains 5 μL of crude extracts prepared from *Cypridina* specimens (6 bodies) in 100 μL of 100 mM glycine-NaOH (pH 7.5). The luminescence reaction was started by adding 1 μL of 1 mM CoA to the reaction mixture. The luminescence activity was determined using a luminometer. Data represents means of I_{max} values from three assays (n = 3).

Fig. S2. Effects of various pH conditions on luminescence intensity of crude extracts from *Cypridina* specimens stimulated with CoA

The crude extracts were prepared from *Cypridina* specimens (11 bodies) in 180 μL of 100 mM ammonium acetate (pH 8.0) and 5 μL of crude extracts in 95 μL of each buffer were stood for 6 h. The luminescence stimulation of crude extracts was determined by adding 1 μL of 100 mM CoA.

Fig. S3. Effects of assay conditions in 100 mM glycine-NaOH at pH 7.5 and pH 8.5 on luminescence intensity of the G25-fraction from *Cypridina* specimens stimulated with PAP and CoA

A. PAP at a final concentration of 10^{-5} M. **B.** CoA at a final concentration of 10^{-3} M. The reaction mixture contains Cypridina luciferyl sulfate (790 ng) in 100 μL of 100 mM glycine-NaOH (pH 7.5 or 8.5). The luminescence reaction was start by adding 5 μL of G25-fraction of *Cypridina* specimens and measured for 60 s, followed by adding 1 μL of PAP (1 mM) or CoA (100 mM), respectively, and luminescence activity was further measured. Arrow indicates the position of adding of PAP or CoA to the reaction mixture.

Fig. S4. Chemical structures of PAP and nucleotide derivatives used in the experiments

Fig. S5. MS/MS analysis of CoA as an authentic compound

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590 **Fig. S6. MS/MS analysis of the reaction product with *m/z* 846 from the reaction**

591 **mixture of CoA, Cypridina luciferyl sulfate, and the G25-fraction**

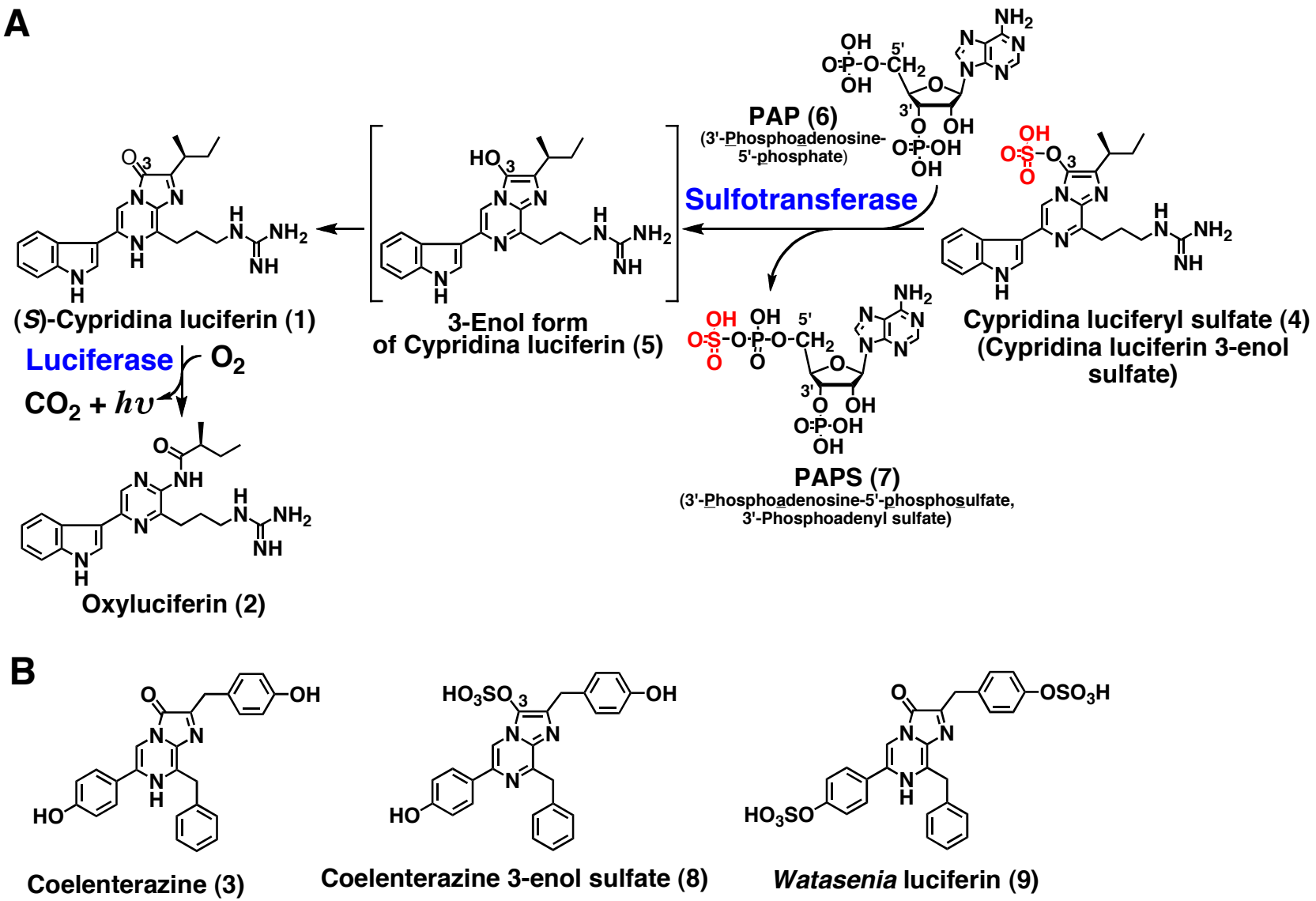
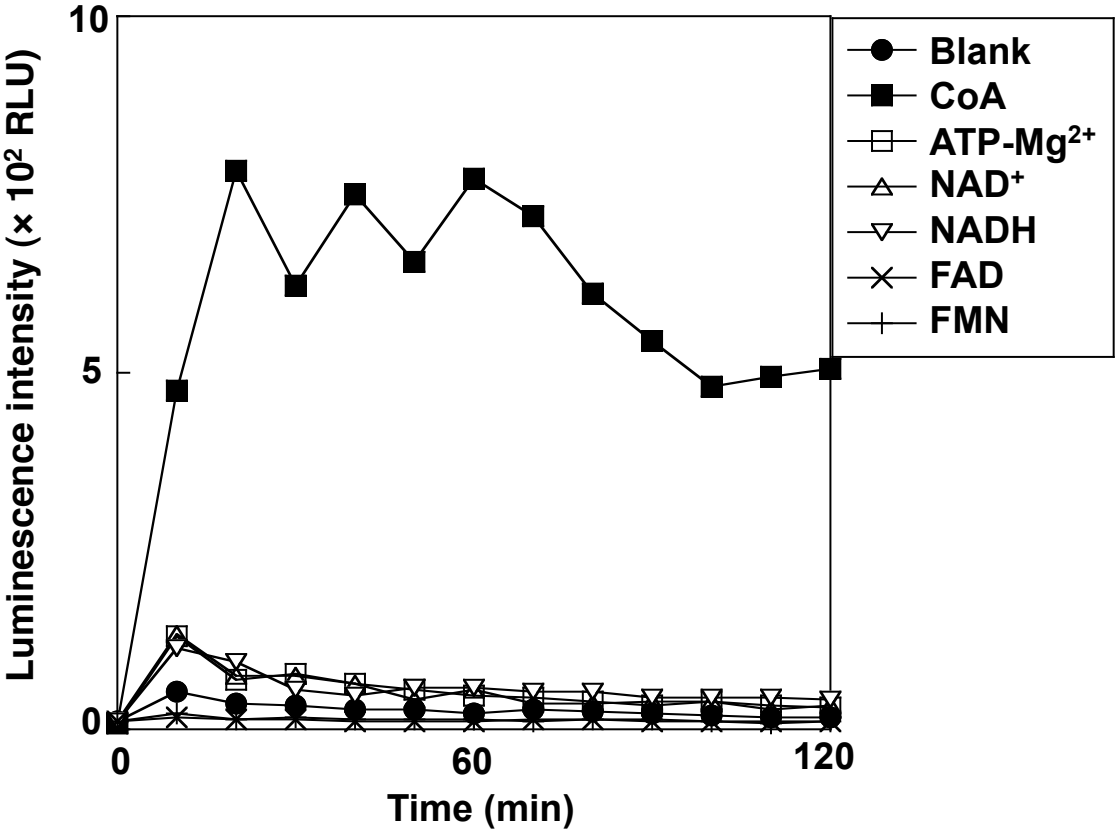


Fig. 1. Nakamura et al.



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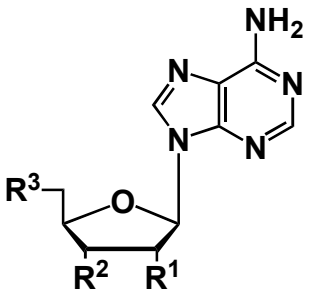
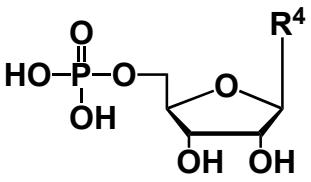
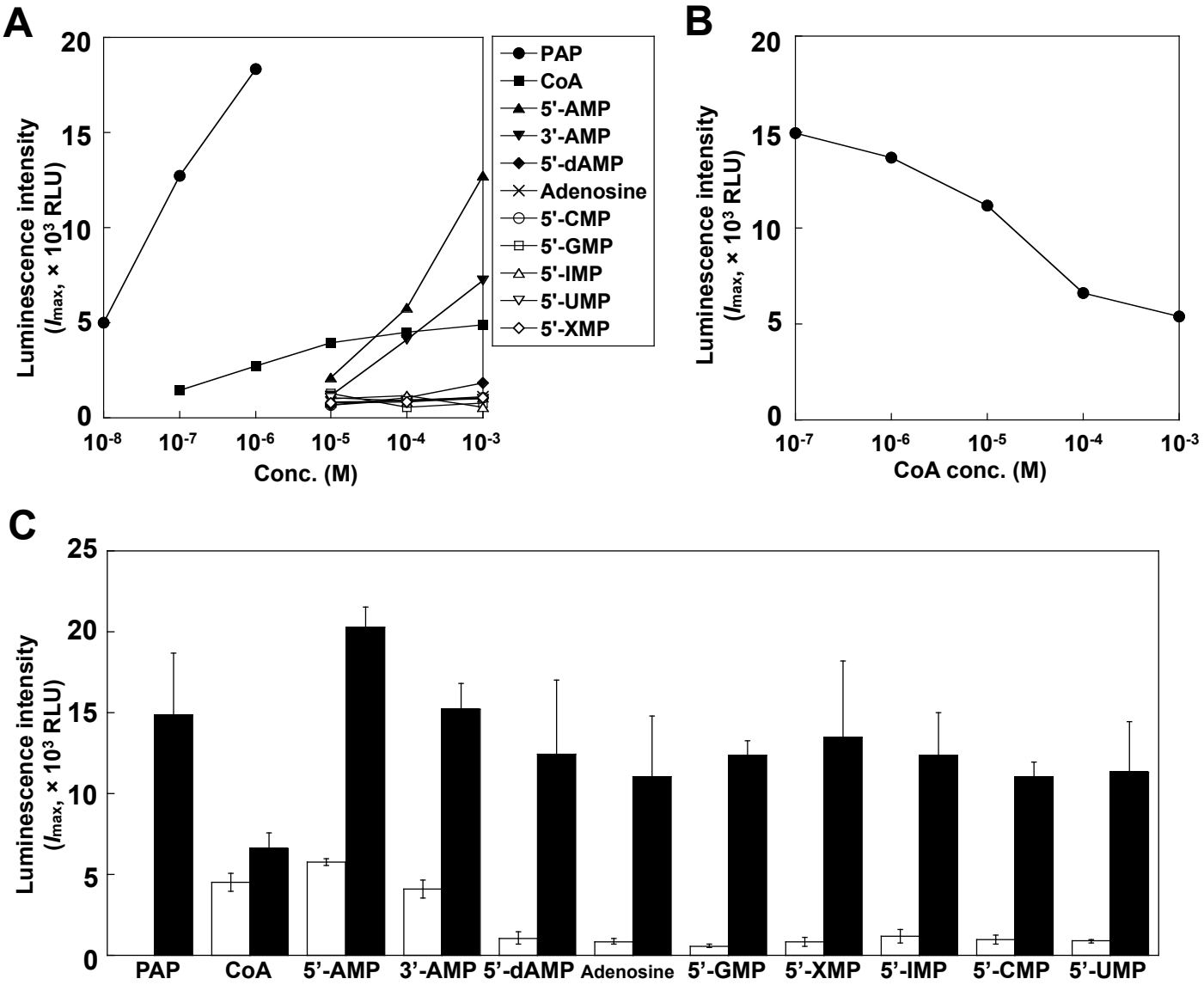




	R^1	R^2	R^3
PAP	OH	OPO_3H_2	OPO_3H_2
3'-AMP	OH	OPO_3H_2	OH
5'-AMP	OH	OH	OPO_3H_2
5'-dAMP	H	OH	OPO_3H_2
Adenosine	OH	OH	OH
Coenzyme A	OH	OPO_3H_2	4'-Diphosphopantetheine

	R^4
5'-AMP	Adenine
5'-GMP	Guanine
5'-XMP	Xanthine
5'-IMP	Hypoxanthine
5'-CMP	Cytosine
5'-UMP	Uracil

Fig. 3. Nakamura et al.



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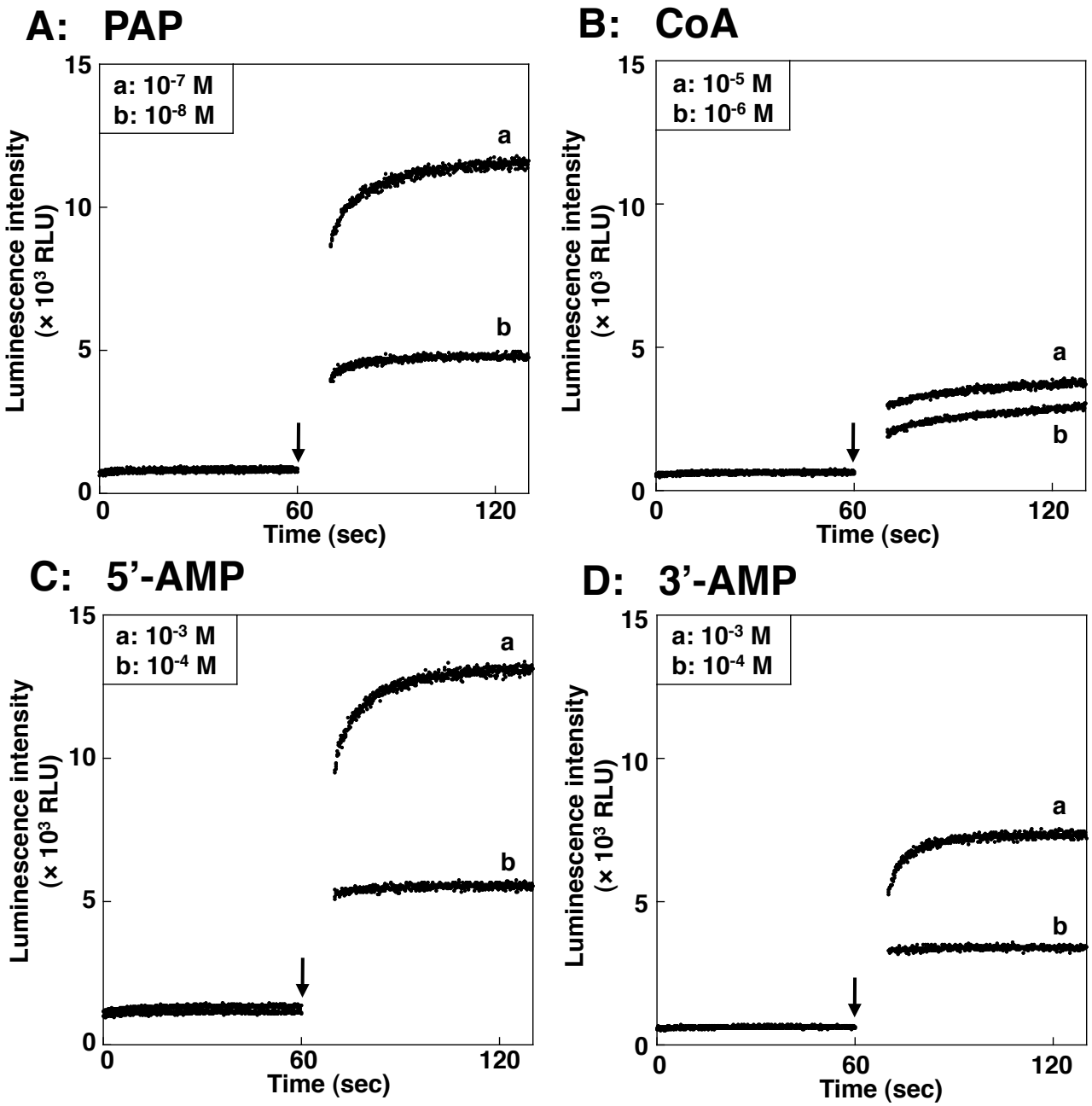

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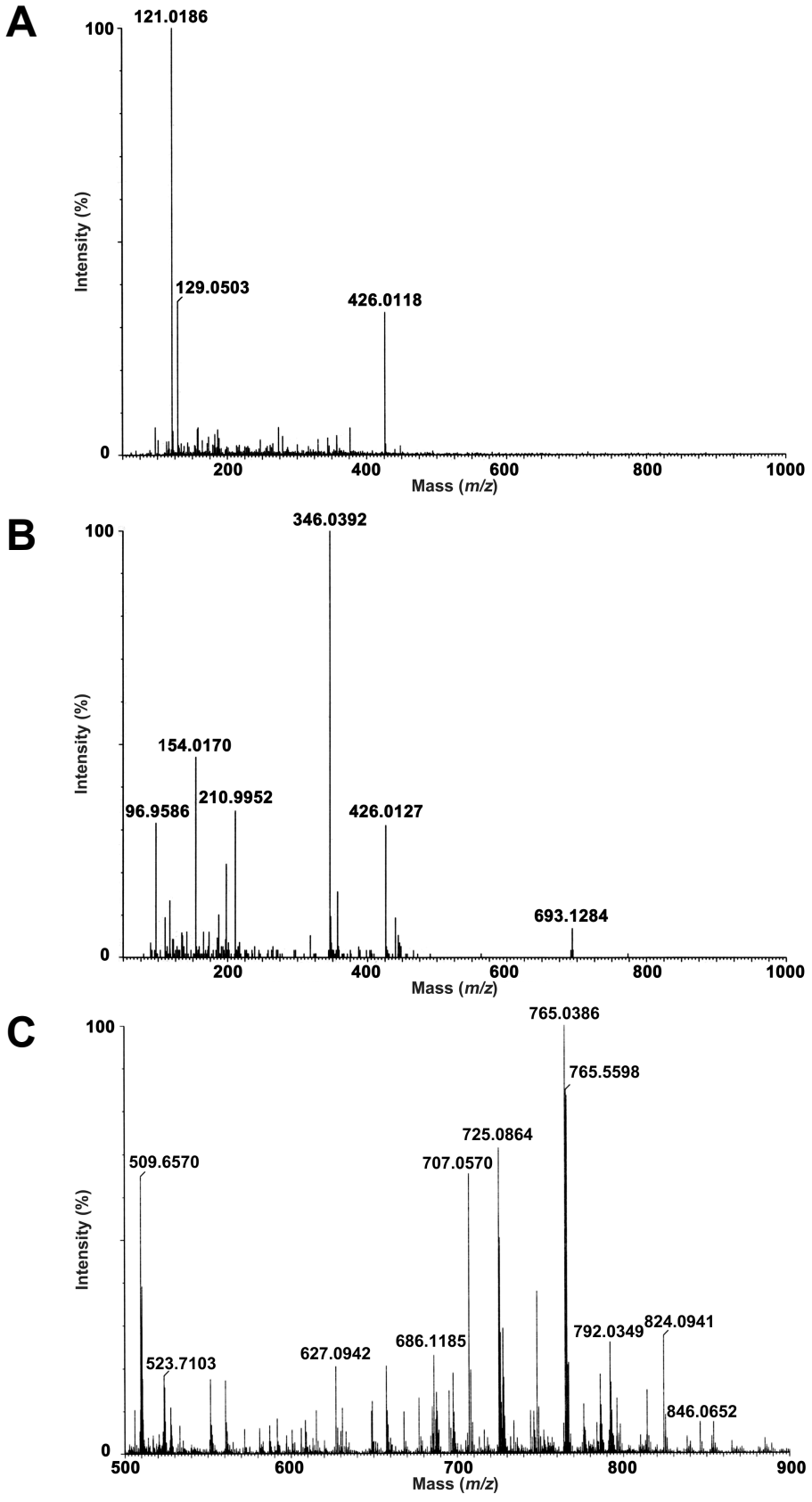

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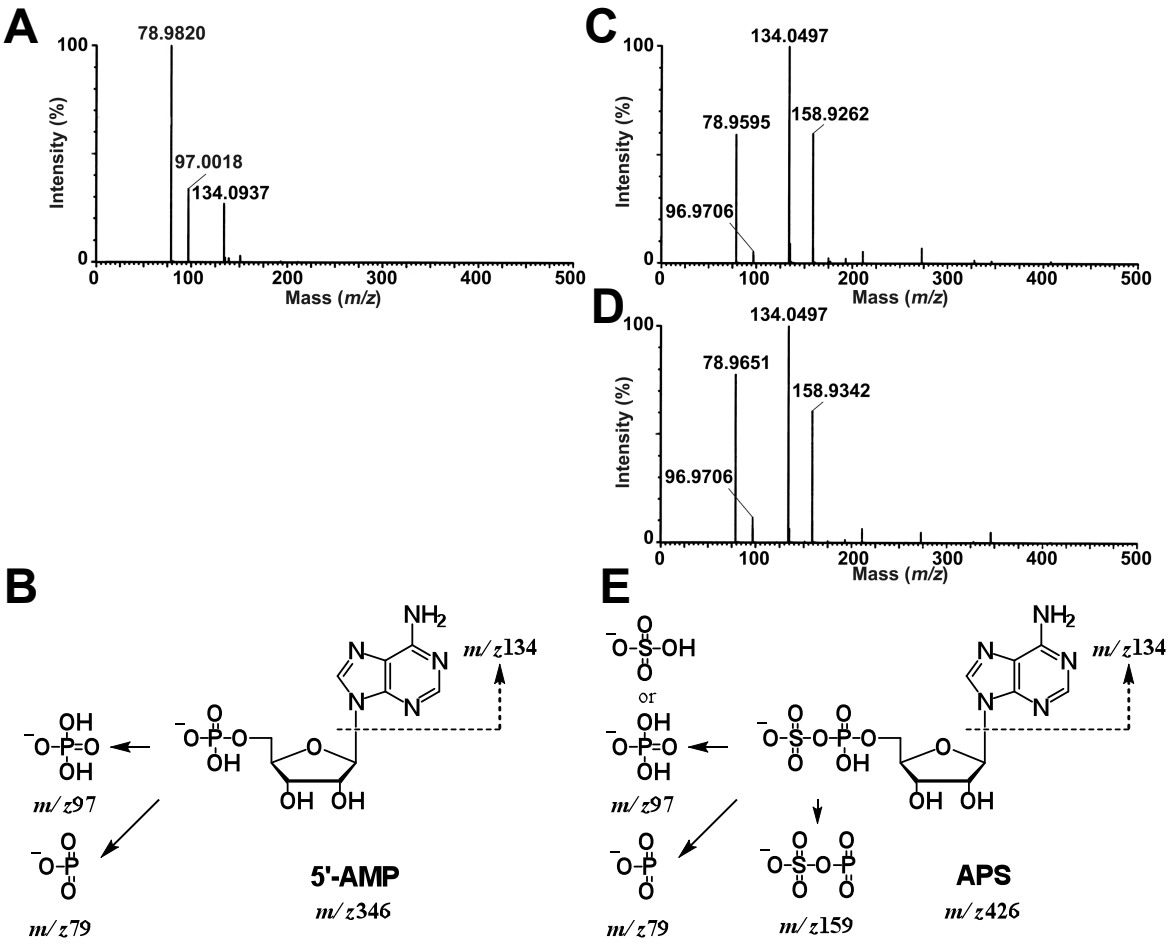

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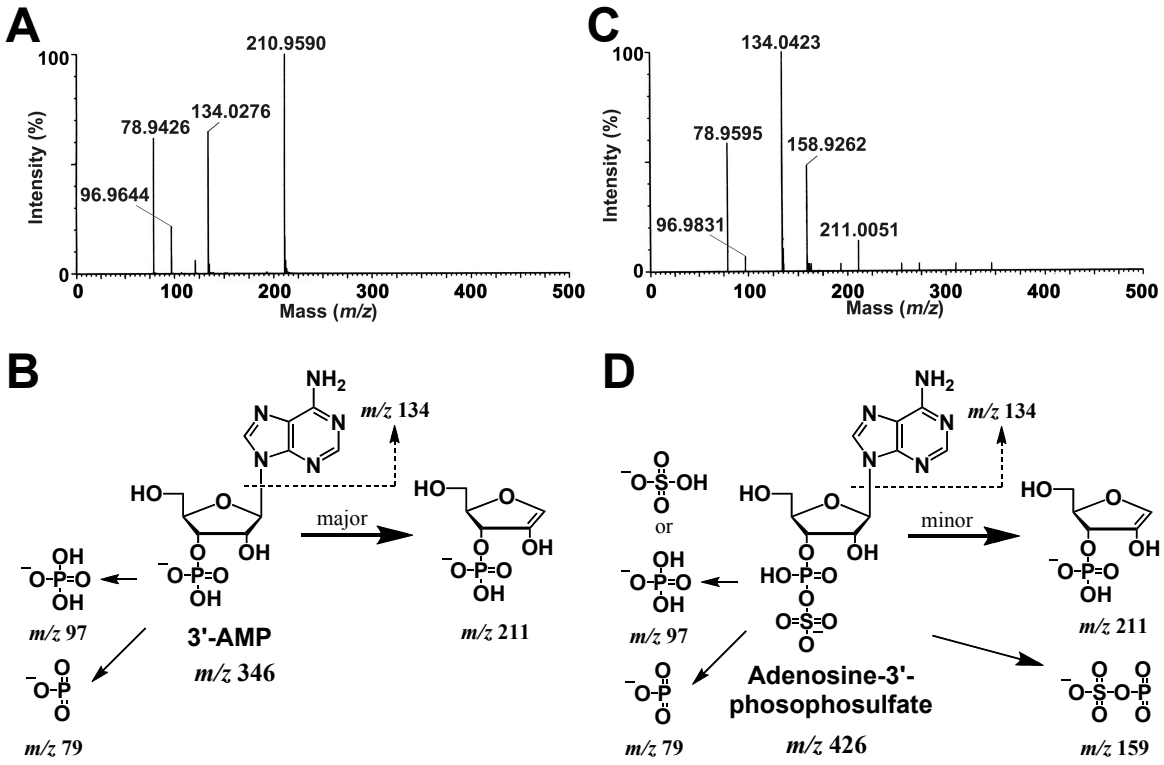

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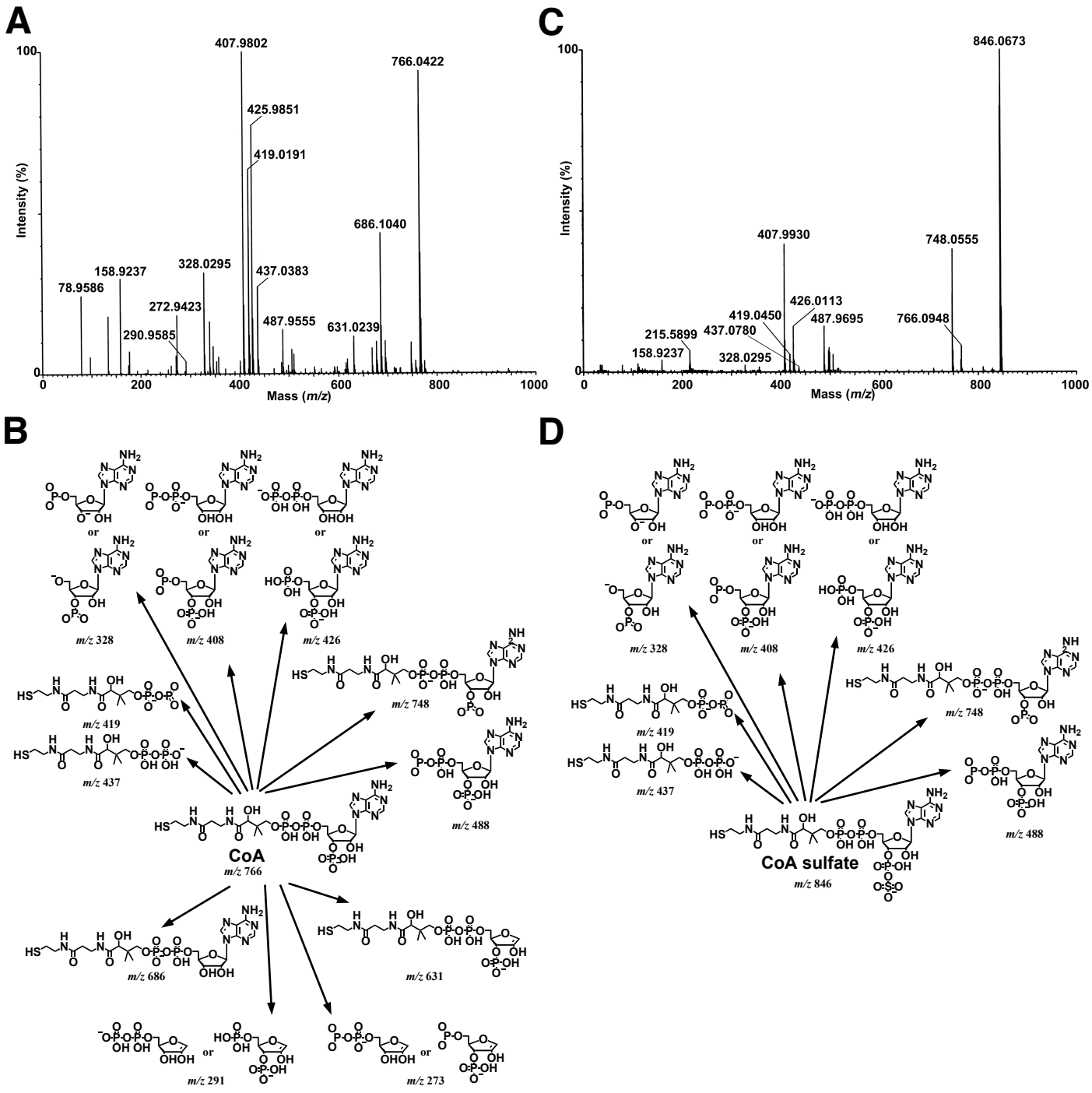

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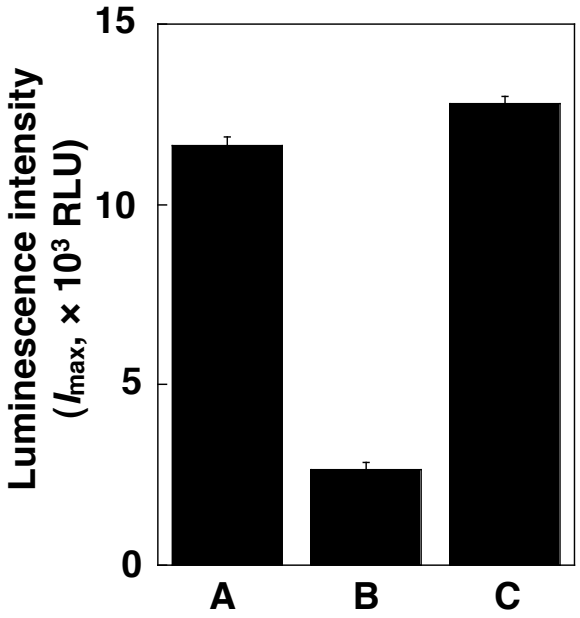

Fig. S1. Purification of CoA from the commercial source and CoA stimulation of luminescence activity in soluble fraction of *Cypridina* specimens

A. CoA (Wako Pure Chemical Industries, Lot No. KWK2017).

B. CoA (Oriental Yeast Co., Lot No. 132605).

C. Purified CoA by HPLC from CoA (Oriental Yeast Co., Lot No. 132605) .

The reaction mixture contains 5 μL of soluble fraction of *Cypridina* specimens (6 specimens) in 100 μL of 100 mM glycine-NaOH (pH 7.5). The luminescence reaction was started by adding 1 μL of 1 mM CoA. The luminescence activity was determined using a luminometer. Data represents means of I_{max} values from three assays (n = 3).

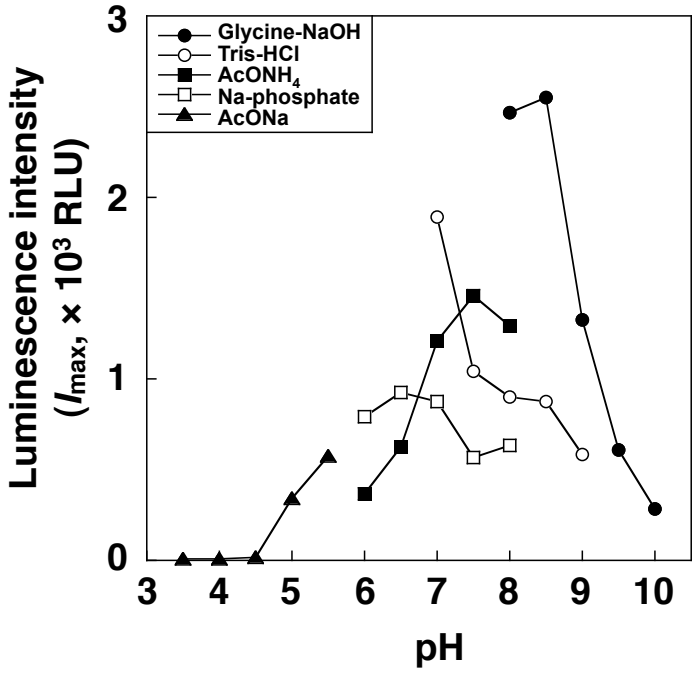

Fig. S2. Effects of various pH conditions on luminescence intensity of soluble fraction from *Cypridina* specimens stimulated with CoA

The soluble fraction was prepared from *Cypridina* specimens (11 specimens) in 180 μL of 100 mM ammonium acetate (pH 8.0) and 5 μL of soluble fraction in 95 μL of each buffer were stood for 6 h and the luminescence stimulation was started by adding 1 μL of 100 mM CoA.

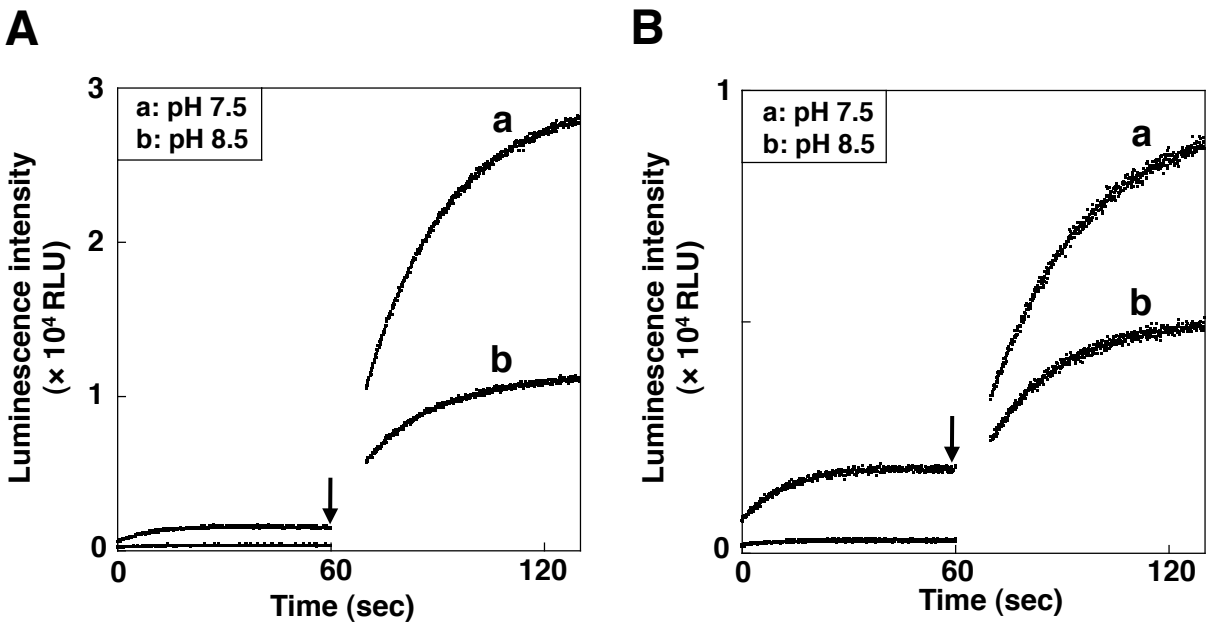

Fig. S3. Effects of assay conditions in 100 mM glycine-NaOH at pH 7.5 and pH 8.5 on luminescence intensity of the G25-fraction from *Cypridina* specimens stimulated with PAP and CoA

A. PAP at a final concentration of 10^{-5} M. **B.** CoA at a final concentration of 10^{-3} M.

The reaction mixture contains Cypridina luciferyl sulfate (790 ng) in 100 μL of 100 mM glycine-NaOH (pH 7.5 or 8.5). The luminescence reaction was start by adding 5 μL of G25-fraction of *Cypridina* specimens and measured for 60 s, followed by adding 1 μL of PAP (1 mM) or CoA (100 mM), respectively, and luminescence activity was further measured. Arrow indicates the position of adding of PAP or CoA to the reaction mixture.

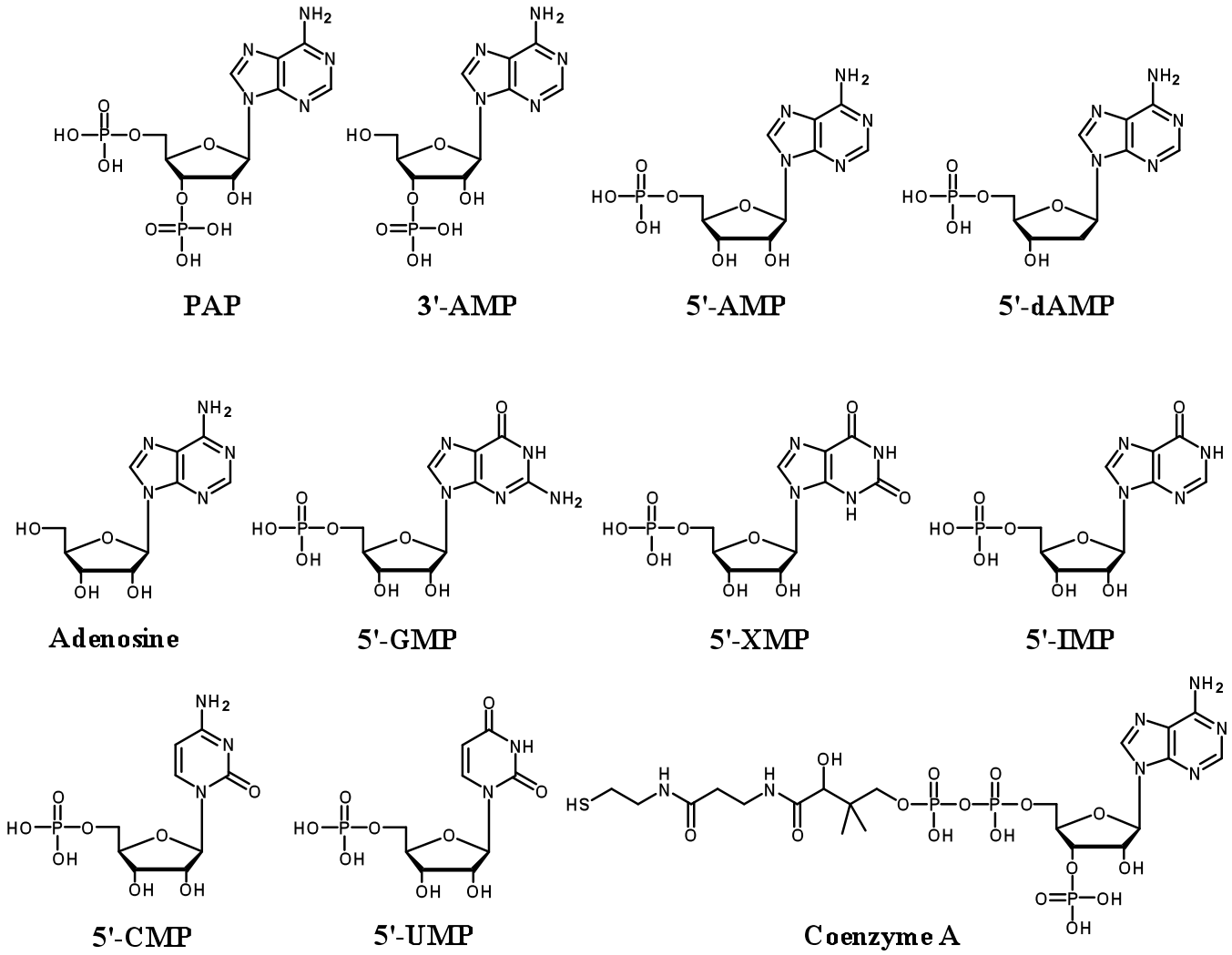

Fig. S4. Chemical structures of PAP and nucleotide derivatives used in the experiments

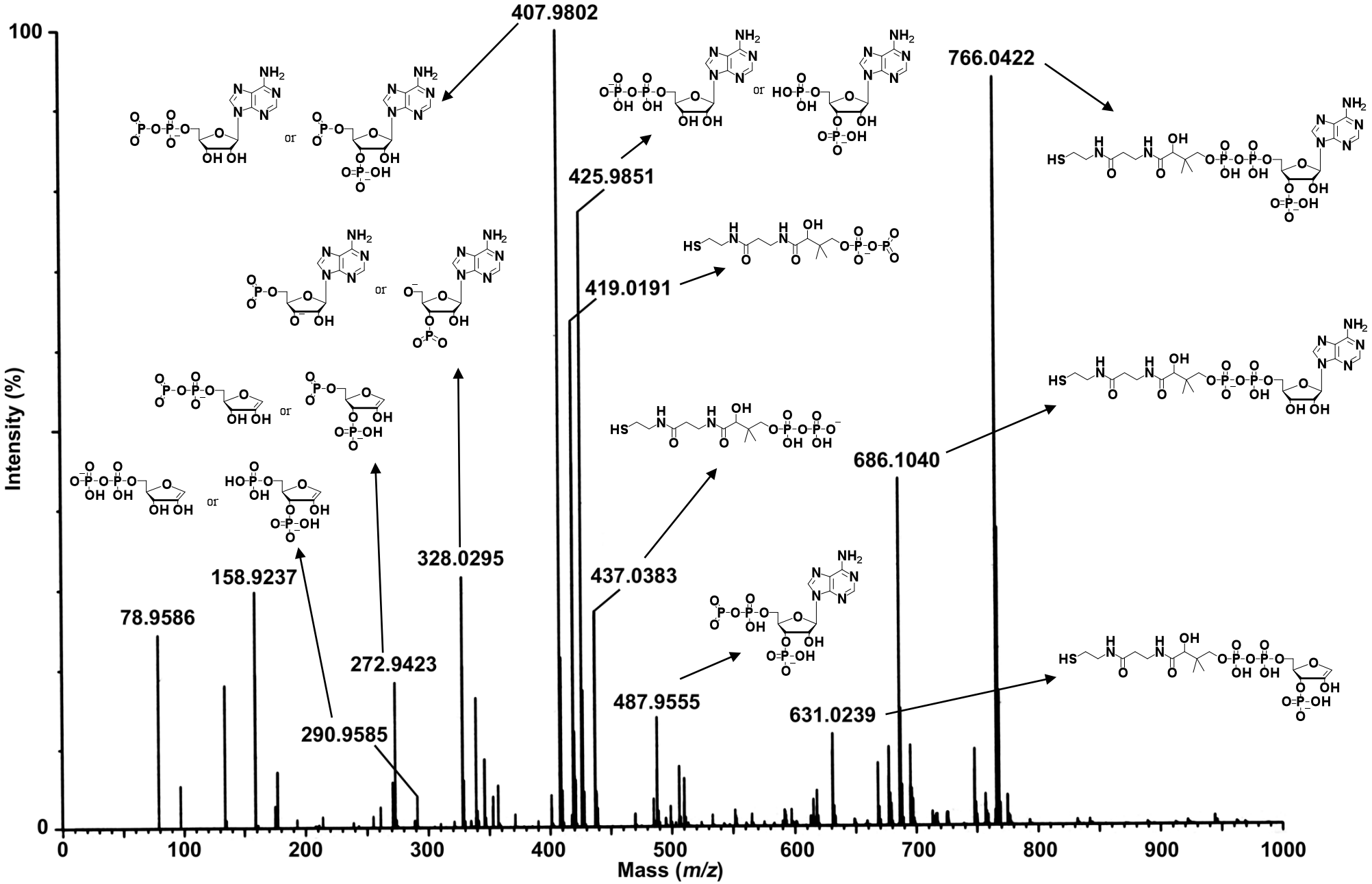

Fig. S5. MS/MS analysis of CoA as an authentic compound

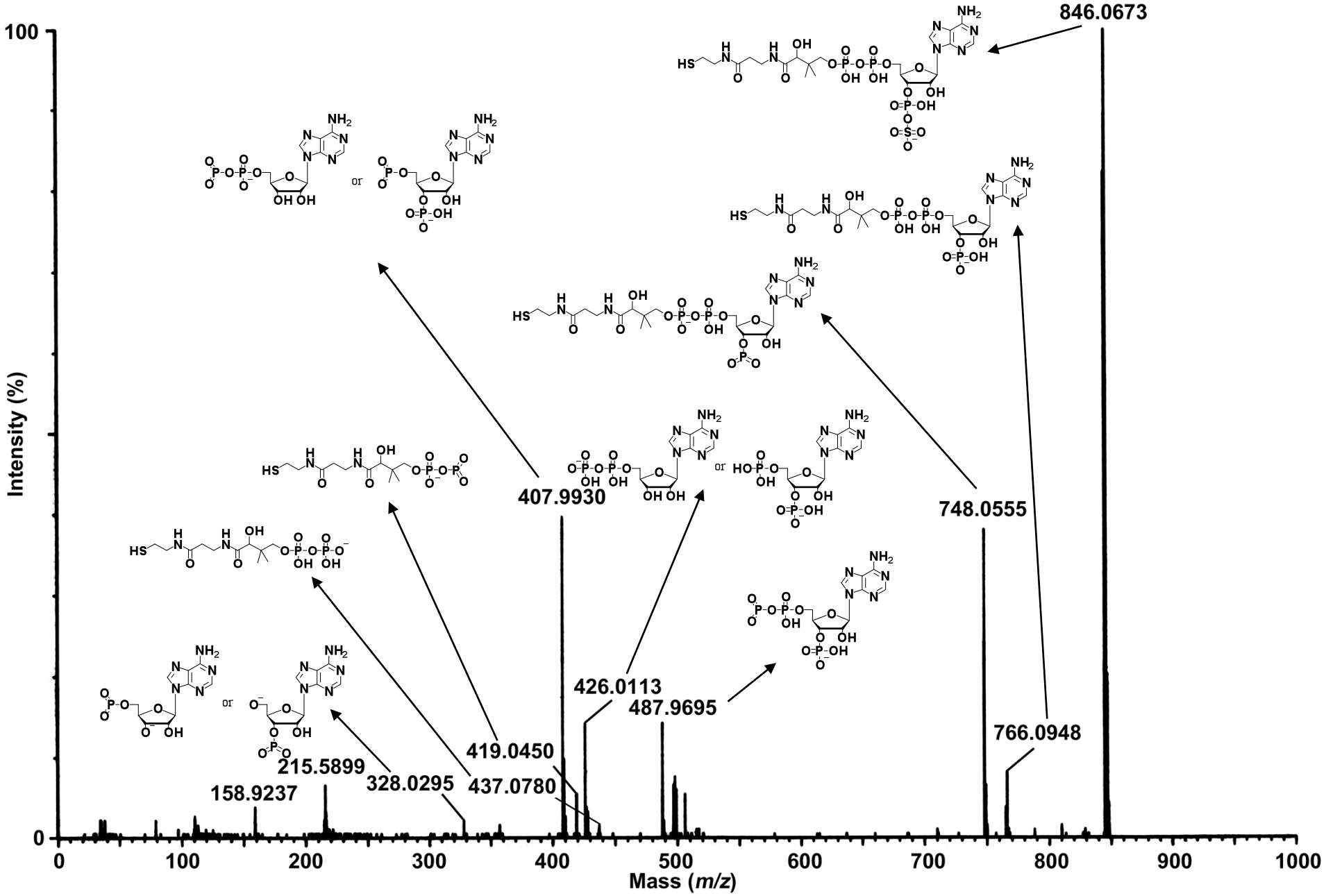

Fig. S6. MS/MS analysis of the reaction product with *m/z* 846 from the reaction mixture of CoA, Cypridina luciferyl sulfate, and the G25-fraction