Synthesis of Alkylated Aminocoumarins with Methyltransferases and Immobilization of SAM

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Danksagung

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Diese Arbeit widme ich meiner Uroma und meinem Opa.

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1. Introduction

One of the most occurring small functional groups in small-molecule drugs is the methyl group. Introduction of this alkyl fragment in biologically active molecules is a special focus in the field of medicinal chemistry and of interest for pharmaceutical industry. More than 67% of the top-selling drugs of 2011 include at least one methyl group. The presence of a methyl group increases the potency of the molecule as a binder from three- to a tenfold boost in a special case which is explained by desolvation effects. Even a simple replacement of C-H to C-Methyl (Me) improves the IC_{50} to a value up to 590-fold in a special case. These observations are two examples of the so called "magic methyl effects" in the medicinal chemistry community.[1]

Not only in drug synthesis methylations are carried out. Methylation is a common reaction in nature. In biological systems the installation of methyl groups is a widely used chemical modification. As already known from biocatalytic transformations, enzymes are very efficient and environmentally benign catalysts.[2]

One big disadvantage for the utilization of enzymes is the need of stoichiometric amount of the cofactor. In this study methyltransferases are used which are cofactor dependent enzymes. The most prevalent cofactor of the biological methylation reactions catalyzed by methyltransferases (Mtases) is *S*-adenosyl-L-methionine (SAM) (Figure 1). The utilization on preparative scale is highly restricted because of the high costs of this natural methyl donor. There is potential in the development of a recycling system for this cofactor.

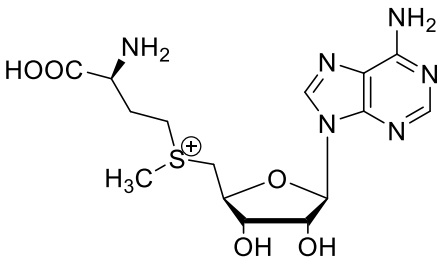


Figure 1: *S*-Adenosyl-L-methionine (SAM).

The high reactivity of SAM is a big benefit for the utilization as methyl donor in methylation reactions.[3] The cofactor scope of methyltransferases can be increased. Several studies have shown that the transfer of different alkyl groups next to SAM is possible. From artificial SAM analogs, not only the methyl group, but also alkyl groups can be transferred to the respective substrates catalyzed by methyltransferases.

2. Theoretical part

2.1. Friedel-Crafts reaction

In the year 1877 the French chemist Charles Friedel and the American mining engineer James Mason Crafts discovered the electrophilic aromatic substitution reaction (S_EAr), the Friedel-Crafts reaction. They found out that by using Lewis acids and halogen alkanes together with benzene an alkylated benzene and halogen hydride occured. The formation of a carbon-carbon bond is one of the most challenging reaction classes in the synthetic chemistry. From that time on it was the method of choice to alkylate aromatic or heteroaromatic compounds till today.[5, 6]

The electrophilic substitution starts with activation of the halogen alkane by the Lewis acid, followed by an electrophilic attack from benzene on the positively charged carbon-ion. At this stage the aromaticity of the benzene is interrupted because of the building of a δ-bond with the activated species. The last step is the deprotonation of the proton on the positively charged benzene and so the aromatic system gets regenerated (Figure 2).[6 - 8]

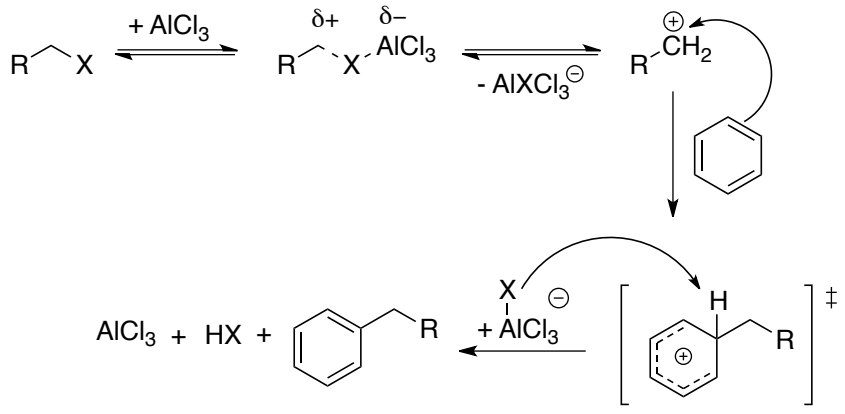


Figure 2: Mechanism of the Friedel-Crafts reaction.

The reaction also bears few drawbacks. The regioselectivity of the reaction is low thus multi alkylations are possible. This is due to the fact that alkylation arises with increased electron density and by introducing alkyl moieties a more nucleophilic product is build. Due to the increased nucleophilicity more alkyl groups can be substituted easily. A mixture of products are obtained,

which are very difficult to separate and the yield is reduced considerably. A second drawback is the rearrangement of the carbenium ions. This leads to formation of side products.[6 - 8]

Over the last years several improvements of the reaction have been achieved. Beside the Lewis acids including $AlCl_3$ also Brønsted-acids for instance sulfuric acid and super acids such as $HF*SbF_5$ are used to accelerate the transformation. A big issue of using stoichiometric amounts of Lewis or Brønsted acids and toxic alkyl halides is the formation of vast amounts of salt side products. In this area a lot of research has been done and there are already some methods whereby metal or acid catalysts can be applied in catalytic amounts.[9]

To make the reaction more environmentally benign, substrates were tested in order to make this process more harmless. The most environmentally friendly compounds which can be used in Friedel-Crafts alkylations to convert arenes and heteroarenes are Ag(I)- and Mo(II)-catalysts.[10] The method was developed by Bandini *et al.*[11] Zhou *et al.* developed a very similar Fe(III)-catalyzed intramolecular Friedel-Crafts alkylation of propargyl alcohols. [9, 11]

In the year 2007 Nishibayashi and co-workers published the first catalytic enantioselective Friedel-Crafts alkylation with propargyl alcohols as electrophiles. They could obtain excellent enantioselectivities up to 94% by using an optically active complex, which induced the chirality.[12]

Allylation

Allylation reactions act via nucleophilic substitution. There is no distinct boundary for secondary alkyl compounds whether these are reacting via S_N1 or S_N2 mechanism, whereby methyl and primary alkyl compounds are clearly reacting via the S_N2 mechanism.[5]

Catalyzing the Friedel-Crafts allylation by transition metals via a metal-coordinated allyl cation linear and branched products can be built (Figure 3).[9]

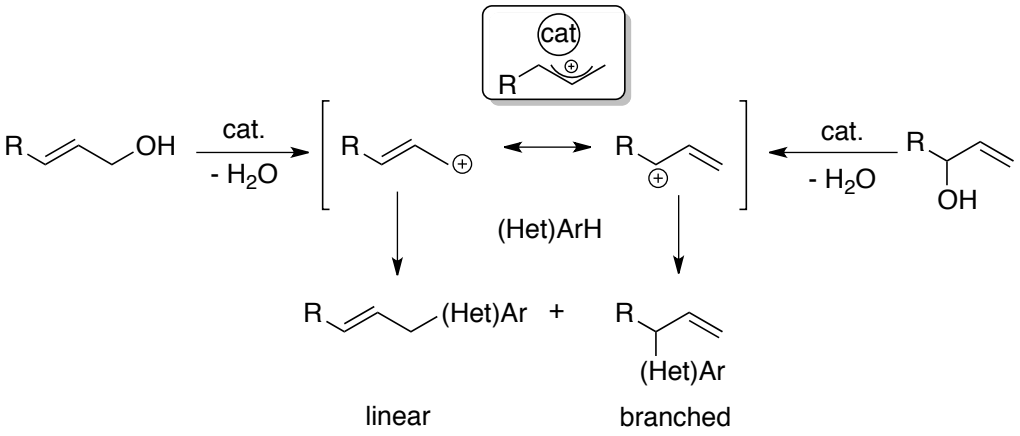


Figure 3: Friedel-Crafts alkylation with allyl alcohols as alkylating reagents.[9]

The first catalytic Friedel-Crafts alkylation of arenes with allyl alcohols was performed 1999 by Kočovský *et al.* The approach was to use small amounts of a Mo(IV)-complex to substitute allyl alcohols with electron-rich arenes such as phenol and anisol. By using $Mo(acac)_2(SbF_6)_2$ a C-allylated arene results and by applying $Mo(acac)_2Cl_2$ an O-allylated phenol was built as major product.[13]

Another working group (Chan *et al.*) engineered an efficient Au(III) catalyst to alkylate cinnamyl alcohols and 1-arylated allyl alcohols. With this method it is possible to use various transition metals (Au(III), Ag(I), In(III), Zn(II) and Cu(II) salts) and Brønsted acids to catalyze the transformation. By using 5% of $AuCl_3$ as catalyst and by running the reaction at ambient temperature good yields of allylated arenes and heteroarenes could be achieved after short reaction times.[14]

Tamura *et al.* developed Friedel-Crafts allylation by using $Pd(PPh_3)_4$ as well as BEt_3 as catalytic systems and allyl alcohols as alkylating agents. In contrast to the reactions described above where a free allyl cation is the reactive allylating species, the Pd catalyzed reaction proceeds by an electrophilic Pd-allyl complex as reactive allylating species.[15]

Methylation

For attaching the simplest alkyl group methyl to an aromatic system effectively other methods than the Friedel-Crafts alkylation is necessary. In these methods first a formylation is performed and then the aldehyde is reduced to the corresponding alkane by the Clemmensen or Wolff-Kishner reductions which are not compatible with many functional groups.[5] Milder conditions would be necessary such as Mozingo reduction.

Methylation of an aromatic ring is possible when alkyl groups are already present in the educt, which increases the nucleophilicity of the ring and electrophilic substitutions are more likely to occur.

2.2. Enzymes

In the nineteenth century the discipline of enzymology, the study of enzymes, evolved. The first records of experiments in this discipline have been found within the year 1810 from Joseph Gay-Lussac. He figured out that by decomposition of sugar by yeast two products result, EtOH and CO_2 respectively. Louis Pasteur suggested in the mid-nineteenths that fermentation process could only occur in living cells. At the end of the century the alcoholic fermentation was named after Eduard Buchner, who established a cell-free yeast extract which could carry out the synthesis of EtOH from glucose.[16]

As already mentioned in the introduction enzymes are very efficient catalysts. Comparing biocatalyzed reactions with chemically catalyzed reaction, the enzyme-mediated processes are by a factor of around 10^4 faster. Enzymes are not only completely biodegradable, but they also work under mild conditions. A big challenge in large-scale applications is the compatibility of enzymes.[2]

Due to the elucidation of the mechanism of enzymes in 1960, we know that enzymes act with a so-called induced-fit mechanism. This means that enzymes are not rigid, they have a soft structure whereby they can change

their conformation under the influence of the substrate structure so as to wrap itself around the whole molecule. This phenomenon leads to the fact that enzymes might not be restricted to their natural substrates. Enzymes show usually high chemoselectivity next to regio- and diastereoselectivity as well as enantioselectivity.

Due to the wide range of reactions that can be performed by different enzymes, biocatalysis is of particular interest for research as well as industry. Classification of enzymes according to the reaction they catalyze is as following:

Oxidoreductase	EC 1
Transferases	EC 2
Hydrolases	EC 3
Lyases	EC 4
Isomerases	EC 5
Ligases	EC 6

The cofactor dependency is associated with large costs and numerous problems. Whereby efficient recycling methods for some cofactors has been developed.

Methyltransferase (Mtase)

Transferases belong to the type of enzymes, which are able to transfer functional groups from a donor to an acceptor molecule. The group of Mtases belongs to the class of transferases with the EC [2.1.1.].

Mtases participate in many biologic transmethylation reactions and are of particular importance. This class of enzymes catalyzes the transfer of a methyl group from a cofactor for example *S*-adenosyl-L-methionine (SAM) (Figure **1**) to the substrate on a C-, S-, N- or O-target atom. At the beginning of the investigation on SAM, it was believed that only small molecules are

methylated.[17] But it turned out that even larger molecules such as DNA, RNA, proteins, lipids and polysaccharides are methylated in the nature. The classification of the Mtases are done due to the substrate and on the type of the acceptor atom.[17]

As already mentioned above Mtases need a cofactor as methyl donor for the alkylation reactions. SAM as cofactor was first described by Cantoni in 1950.[18] SAM is the most commonly used cofactor, next to methyltetrahydrofolate, methylcobalamin and betaine in nature.[17]

SAM shows several chemical advantages in comparison to other methyl donors. The high reactivity of SAM is due to the fact, that the methyl group is bound to a charged sulfur atom at the SAM molecule, which thermodynamically destabilizes the molecule and is very reactive.[3] The synthesis of SAM needs a lot of energy. It is the only reaction known to date that requires hydrolysis of all three phosphates from ATP to attach their adenosine moiety together with methionine to build SAM (Figure 4).[17]

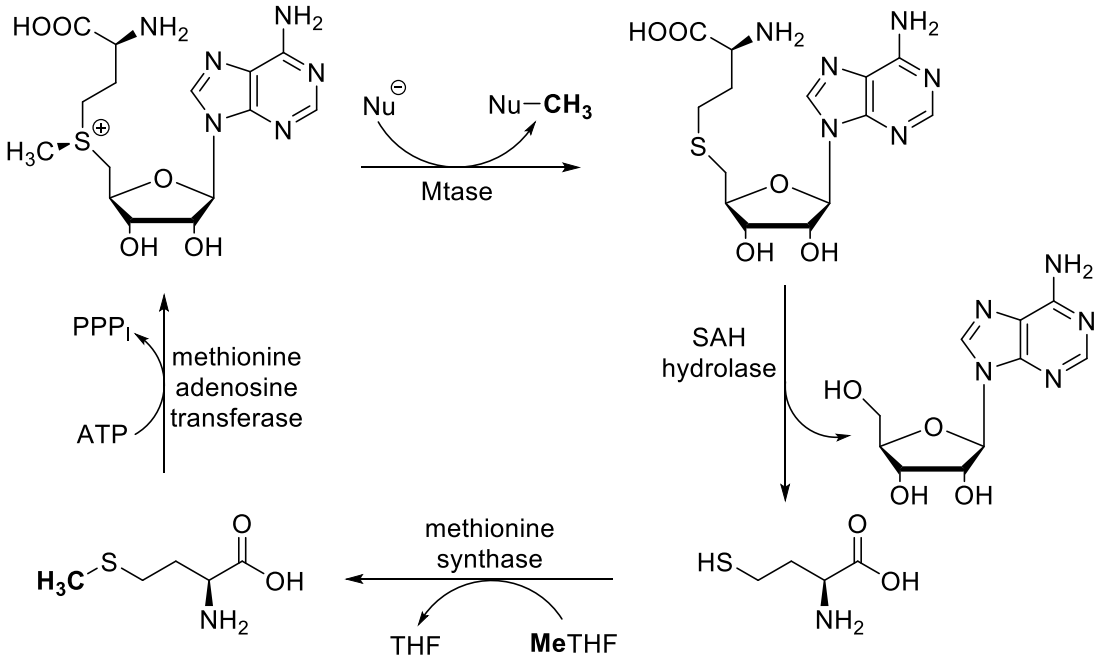


Figure 4: Activated methyl cycle.

The transmethylation reaction of Mtases with SAM is thought to proceed via an S_N2 mechanism with inversion of configuration at the transferred methyl group (Figure 5).

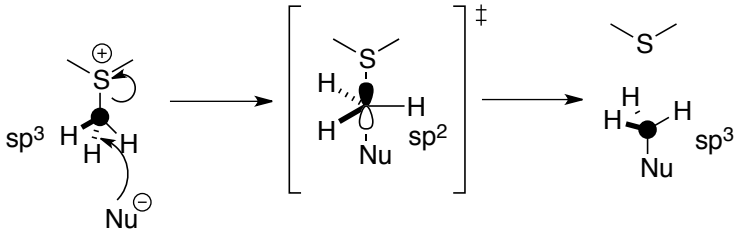


Figure 5: S_N2 reaction of Mtases with SAM.[22]

Aminocoumarin C-Methyltransferases

In this work, a C-Mtase is used, more precisely the aminocoumarin C-Methyltransferase CouO. The antibiotic coumermycin A_1 is shown in Figure 6.[20]

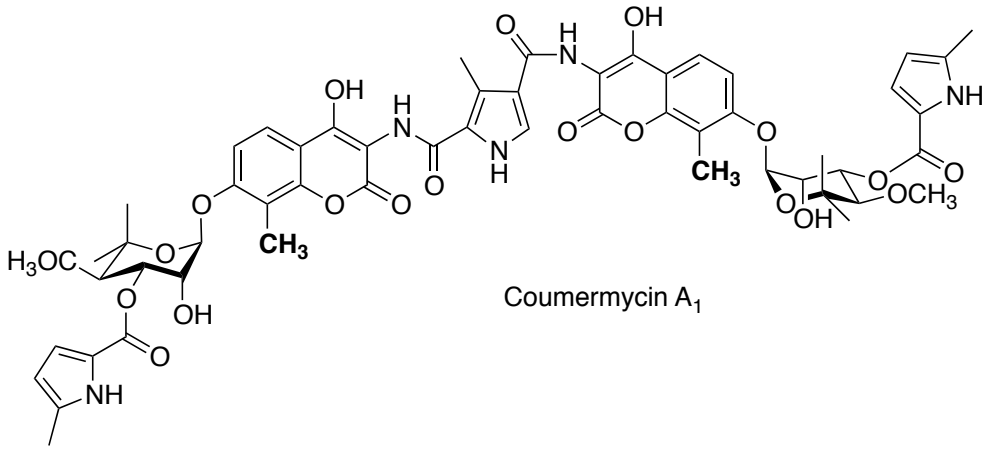


Figure 6: Structure of coumermycin A_1.[20]

The enzyme CouO is involved in the biosynthesis of the aminocoumarin coumermycin A_1 in *Streptomyces rishiriensis*.[21] The target of coumermycin A_1 is the bacterial DNA gyrase. By X-ray crystallographic analysis it was possible to determine the essential functional groups of the substrate in the inhibition of gyrase. Up to 50% inhibition with the antibiotic coumermycin A_1 of the gyrase was obtained.[20] The aminocoumarin antibiotics are all potent inhibitors of the DNA gyrase.

Analogues of *S*-adenosyl-L-methionine

In the 1980s Schlenk and Dainko showed the biosynthesis of SAM cofactor analogues. It turned out that ethyl or propyl groups instead of the methyl group in SAH make the allyl transfer reaction a lot slower.[23] Several years later Weinhold *et al.* synthesized several SAM analogues chemically. The chemical synthesis leads to a 1:1 diastereomer mixture. It is possible to purify the mixture for example by preparative HPLC to obtain the preferred S-epimer.[19]

In the synthesis of SAM analogues is carried out at ambient temperatures and under acidic conditions to avoid alkylation of amino groups of the substrate. The reaction is carried out with SAH as limiting component, with a big excess of alkylating agents (Figure 7).[24]

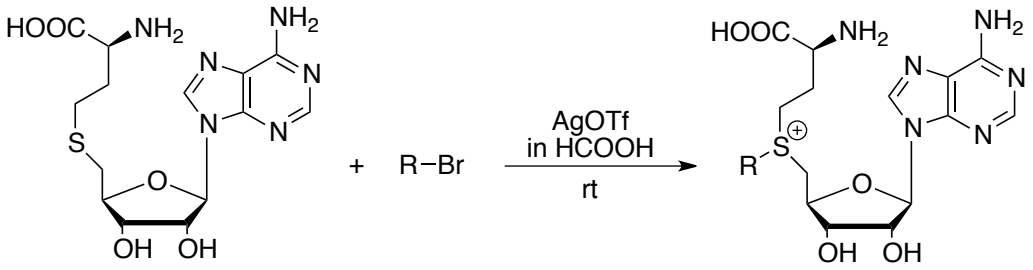


Figure 7: Synthesis of SAM analogues according to the modified procedure of Stecher *et al*.[4]

It is possible to alkylate coumarin compounds with SAM analogues catalyzed by Mtases, NovO and CouO (Figure 8). The reaction is carried out at ambient temperatures with the C-Mtases NovO and CouO. This biocatalytic reaction leads to results up to 99% conversion of the educt.[4, 25]

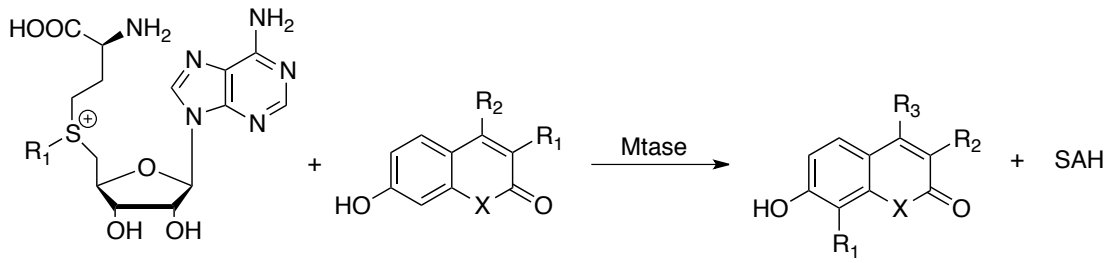


Figure 8: Synthesis of alkyl coumarin derivatives.[4]

It has been shown that the leaving group of methyltransfer reactions SAH is a very effective inhibitor of the protein methylase II.

2.3. Cofactor immobilization

The recycling of cofactors is an ongoing-problem in some cases. Intensive research has already been performed to find a way for efficient recycling models for several cofactors. These substrates have different functions in biocatalytic reactions, e.g. carbonyl reduction, alcohol oxidation, alkylation or carbon-carbon ligation need cofactors. Recycling methods are already developed for NAD^+/NADH, $NADP^+$/NADPH and ATP. For the two cofactors SAM and Acetyl-Coenzyme A (Acetyl-CoA) recycling methods are already known, but they are still to complicated to perform efficiently.[2]

Immobilization positions

As already mentioned above there are several positions of SAM, which are potential targets for immobilization on solid supports. The focus is on four different positions to attack the linker for immobilization on solid supports.

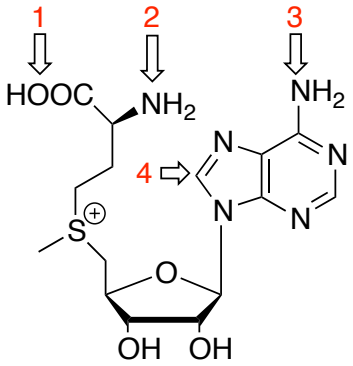


Figure 9: Four potential linkage positions in SAM.

One possible linkage method to immobilize the cofactor SAM is the use of poly(ethylene glycol) (PEG) linker. The advantages of short-chain PEGs or oligo(ethylene glycol)s (OEGs) to use as linker for targeted drug delivery systems are their water solubility, stability to metabolism in biological systems

and their low cost and availability in wide range of molecular weight distributions.[26]

The heterobifunctionality of OEGs with highly reactive end groups is highly appreciated. One end is required for attaching to the solid support and the other one for attaching a targeting ligand, in our case the cofactor SAM. This allows properties such as hydrophilicity, flexibility and biocompatibility to be tuned.[26]

3. Aims

Methylated or alkylated compounds are very important and efficient agents in the pharmaceutical industry. But the synthesis of alkylated molecules is often associated to harsh conditions and combined with the utilization of toxic alkylating agents next to metals, such as Pd, Ru and Rh. Also the low regio- and chemoselectivity is a big drawback in using conventional chemistry conditions. To avoid the selectivity and ecological problems several research groups investigate on enzymes as selective and mild alkylation catalysts.

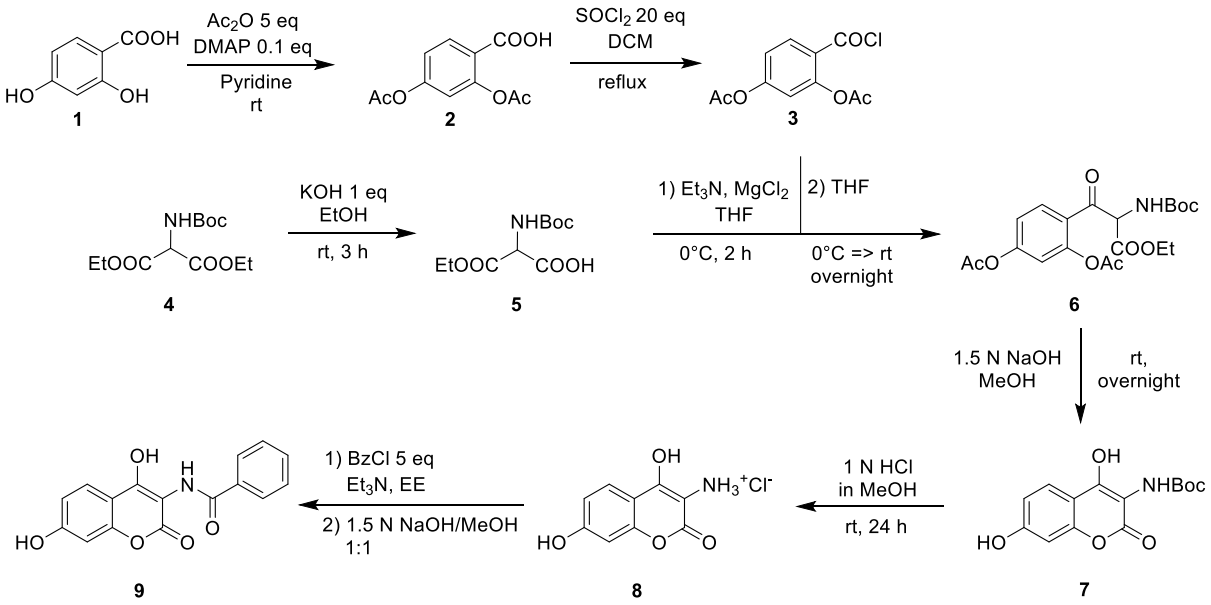
The goal of the work was to synthesize an allyl-analogue of the cofactor SAM in order to transfer the allyl group from the cofactor to an artificial substrate. Before that was possible, it was necessary to find a short and efficient synthesis for the aminocoumarin substrate. The C-methyltransferase CouO was used as catalyst. A strategy for the synthesis of the substrate was previously developed. It was a long methodology and thus it was an important goal to find a short route to prepare the substrate. The characterization of CouO was previously done to find the ideal pH value, temperature and several necessary conditions for the optimal enzyme performance.[4]

A very big drawback by utilization of enzymes in preparative scale is the need of cofactors if cofactors dependent enzymes are needed. Most methyltransferases need the cofactor SAM. The cofactor is very expensive and unstable. No recycling procedure is available.

The second part of this work was to develop an immobilization method for the cofactor SAM. Therefore a PEG linker was synthesized for the attachment on different positions of SAM in order to immobilize the cofactor.

4. Results and Discussion

4.1. Synthesis I of CouBz



Scheme 1: Synthetic route I to *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide **9** for CouO.

The first step of the synthesis was the acetylation of two hydroxy groups in 2,4-dihydroxybenzoic acid **1** in pyridine.[27, 28] The product 2,4-diacetoxybenzoic acid **2** from this overnight reaction was either purified by column chromatography or in the best case crystallized to a white powder. By adding DMAP the reaction was complete within 16 h and high yields could be obtained.

In the next step the carboxyl group of 2,4-diacetoxybenzoic acid **2** was activated to an acyl chloride using thionyl chloride under reflux. 4-(Chlorocarbonyl)-1,3-phenylene diacetate **3** was obtained quantitatively.[28]

To be able to perform the carbon-carbon coupling reaction to synthetize compound **6** diethyl 2-((*tert*-butoxycarbonyl)amino)malonate **4** had to be monosaponified using KOH in EtOH.[29, 30]

During the repetition of the synthesis of compound **5** a new bought batch of the educt **4** did not show any conversion. Although the NMR measurement showed no contamination of the substrate, the more sensitive HPLC analysis showed a peak next to the substrate peak, which was seemingly an interfering impurity. As a

consequence this batch was discarded and a new ordered, which led to the desired product.

The synthesis of compound **6** was achieved in two steps. First compound **5** was activated by deprotonation by Et_3N and stabilized by the Lewis acid $MgCl_2$. After 2 h at 0°C freshly distilled the carbonylchloride **3** was added to the reaction mixture and the product **6** was built during an overnight transformation.[31]

Several problems occurred during the synthesis. The removal of the thionyl chloride excess was very important in order to avoid on one side the reaction with the more active $SOCl_2$ compound and the malonate educt and secondly to capture of the base Et_3N by the acid $SOCl_2$. The reaction was first performed in a Schlenk flask under protective gas (N_2) which did not lead to the desired product. Probably the mixing of the reaction mixture was not effective. After considering all these factors the last optimizing step was to perform the reaction in a two-neck round-bottom flask under protective gas. These optimization studies and adaptions led finally to the desired compound **6**.

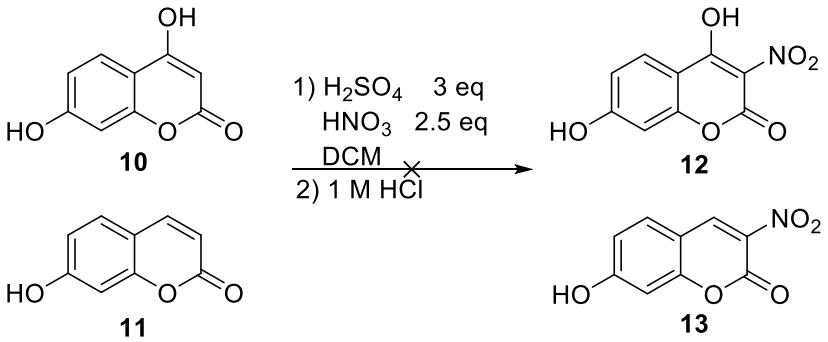
For the ring closure compound **6** was solved in MeOH and then 1.5 N NaOH was added.[31] The conversion of **6** to the product **7** was straight forward, if first MeOH was added to solve the educt and afterwards the 1.5 N NaOH was added. If this was not the case and the base was added first the educt was not soluble anymore, the stirring bar got stuck in the viscous solution.

Removing the Boc protecting group from **7** was performed at very acidic pH.[31] In the best case complete conversion was obtained overnight, but few times it took more than 24 h to prepare compound **8**. In contrast to the description in literature [34] precipitation of the product never occurred. One time the reaction was carried out in a two-phase system under neutral conditions during the workup and the product was found in the organic phase. This reaction could not be reproduced.

The last step of the multistep synthesis was the benzylation of **8** with an excess of benzoyl chloride, which was considered not as critical point of the synthesis. The removal of the excess of benzoic acid, of acylchlorid and the ester cleavage which were built at the two deprotonated hydroxyl groups turned to be problematic. Stirring a prolonged time in basic conditions led to the desired product *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide (CouBz) **9**.

The long synthesis was difficult to perform and at the end very low yields were achieved. The goal was to have enough of the final product CouBz **9** in hands in order to perform activity assays and preparative scale biotransformations. Several repetitions of each step of this synthesis sequence did not lead to sufficient amounts of the product for the forthcoming experiments. Therefore an alternative method had to be developed.

4.2. Nitration of Coumarins



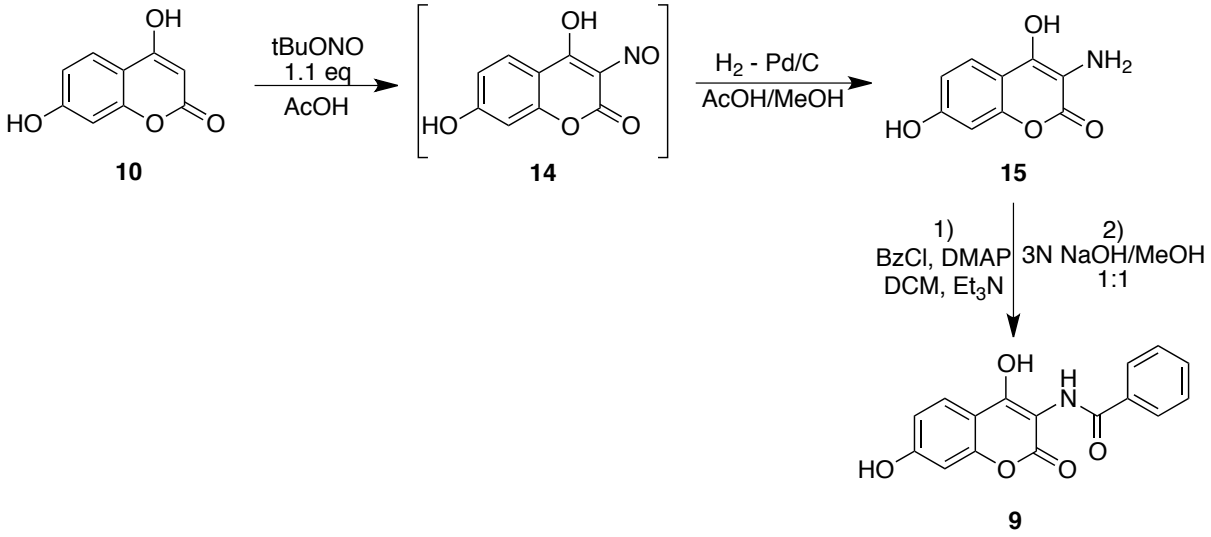
Scheme 2: Nitration of 4,7-dihydroxy-2*H*-chromen-2-one **10** and 7-hydroxy-2*H*-chromen-2-one **11** as precursor compounds for aminocoumarins.

The nitration of the two coumarins **10** and **11** at the C-3 position was carried out under standard conditions by using conc. HNO_3 and conc. H_2SO_4 to give rise to a fuming nitric acid in dichloromethane (DCM).[32]

The reaction was monitored by TLC and new spots were visible. The workup was performed but in contrast to the literature report [32] no precipitation occurred. Also the chromatographic analysis (HPLC) did not show the desired product peaks.

4.3. Synthesis II of CouBz

An alternative procedure is shown in Scheme 3. The novel reaction conditions are the use of *tert*-butyl nitrite (*t*BuONO) as nitrosylating agent and the one-pot reduction of the NO group with 10% palladium on carbon (Pd/C) under H_2-atomsphere.[33]



Scheme 3: Synthetic route II to *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide **9**.

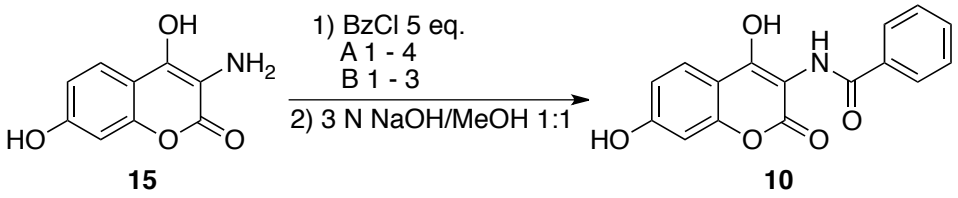
The first step was the nitrosylation of 4,7-dihydroxy-2*H*-chromen-2-one **10** under acidic conditions using *t*BuONO.

The product was not isolated and the reduction with 10% Pd/C under H_2-atomsphere was performed in the same pot.

The benzylation was modified. In contrast to the synthesis described above the catalyst 4-(*N,N*-dimethylamino)-pyridine (DMAP) was added as catalyst. Also the workup conditions were slightly modified. Instead of ethyl acetate (EE) DCM was used for extraction and it turned out to be the better solvent. The choice of an alternative solvent improved the reaction. Already after three steps with just one purification step at the end the new approach led to the desired product *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide (CouBz) **9**.

The very efficient synthesis route (Scheme 3) was much less time-consuming and resource saving than the previous approaches. Just one column chromatography step was needed to purify the product at the end instead of the long synthesis route I with nearly one chromatography step after each stage. Fortunately, due to this synthesis enough substrate could be produced for the enzymatic reactions and activity assays.

Optimization of the last step



Scheme 4: Optimizing the conditions of the amidation step to end product **9** (solvent A and base B).

The last step of the synthesis to build *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide **9** was optimized due to several factors (Scheme 4).

Table 1: Different variants to optimize the acetylation.

No.	Variant	A (solvent)	B (base)	Conversion
1	A1	EE	Et_3N	Yes
2	A2	DCM		Yes
3	A3	MeOH		No
4	A4	DMF		No
5	B1	DCM		Yes
6	B2		K_2CO_3	Yes
7	B3		Pyridine	No

The first investigations have been performed on the solvent for the acetylation step (A1 – A4 in Table 1). Four different solvents EE, DCM, methanol (MeOH) and *N,N*-dimethylformamide (DMF) have been screened. Conversion was obtained in EE and DCM in contrast to MeOH or DMF.

Both solvents should lead to selective acetylation on NH_2 group without attacking the OH groups.[32] One main problem of the reaction was the low solubility of the substrates in these solvents. After several days no conversion was detectable.

The next optimization step was the screening of different bases to deprotonate the NH_2 group for the benzylation (B1 – B3 in Table 1). Three different bases Et_3N, K_2CO_3 and pyridine have been screened, respectively. As already shown before Et_3N operates very well. Even K_2CO_3 is a good base for this step. No conversion was detectable by using pyridine as base.

Table 2: Optimized conditions for the acetylation step.

Variant	A (solvent)	B (base)
A2, B1	DCM	Et_3N

The optimized conditions are summarized in Table 2 with DCM as solvent (variant A2) and Et_3N as base for the acetylation step (variant B1).

4.4. Biocatalysis

Enzyme screening assay

To test the efficiency of the two different CouO lysates “Wild type Alt” (WT A from 2009) and “Wild type Neu” (WT N from 2014), transformations with two substrates were screened. The aminocoumarins CouBz **9** and 2,4-dihydroxy-2*H*-chromen-2-one (Cou_2OH) **10** were used with the conditions, which are shown in Figure **10**.

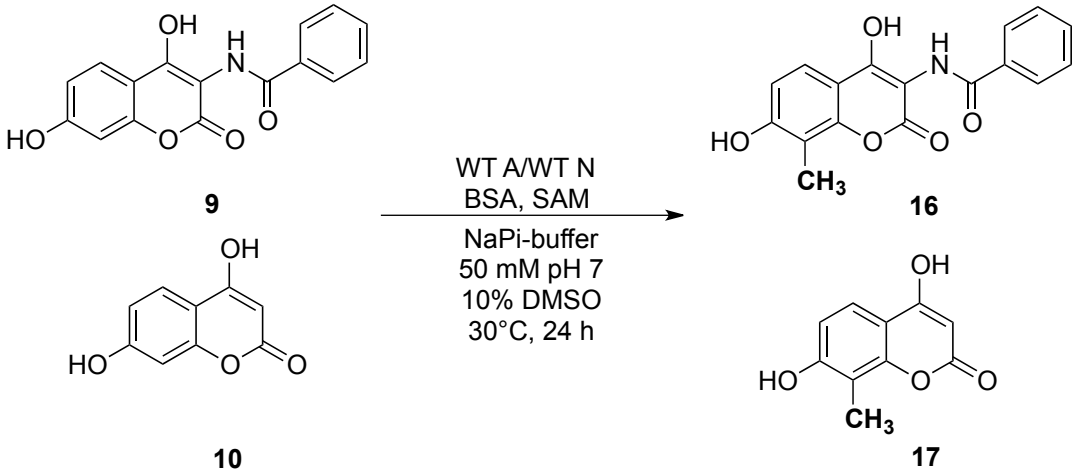


Figure 10: Enzyme assay with the substrates CouBz **9** and 2,4-dihydroxy-2*H*-chromen 2-one **10**.

After 24 h the methylation reactions were stopped by heating the vials 10 min at 80°C and 1000 rpm and the mixture was analyzed by HPLC-MS. The differences between these two enzyme preparations are not significant as shown in Figure 11. The conversion from CouBz **9** to the methylated form *N*-(4,7-dihydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl)benzamide **16** was not complete while Cou_2OH **10** was methylated quantitatively.

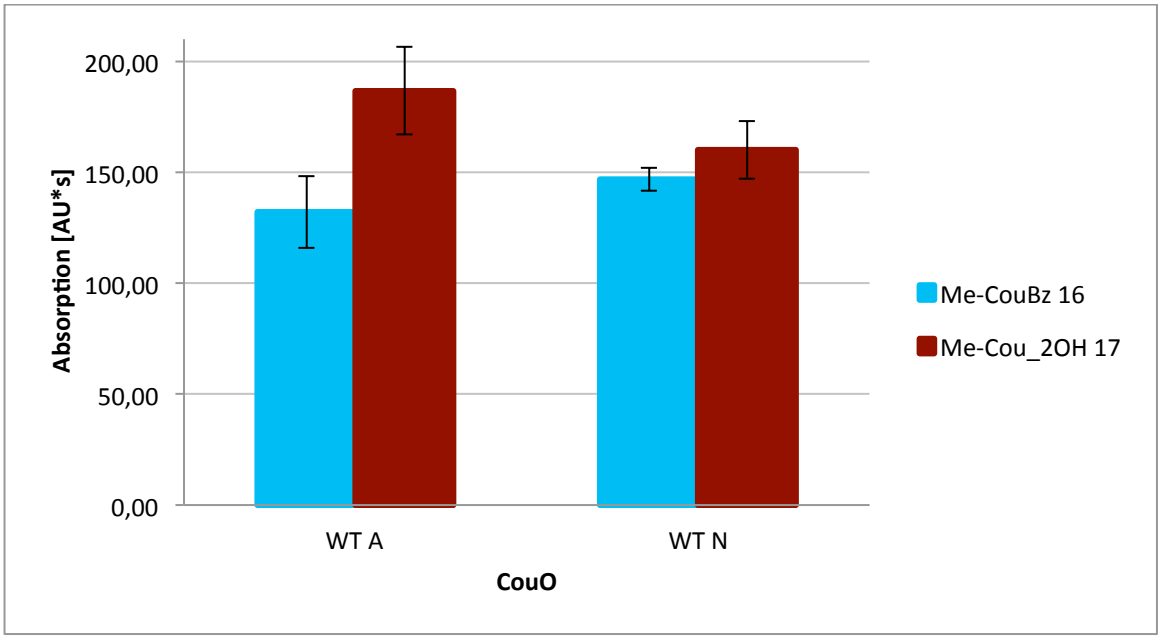


Figure 11: Absorption area of methylated products from the enzyme assay with WT A and WT N.

For the preparative scale experiments the WT A was used.

Synthesis of Allyl-SAH 19

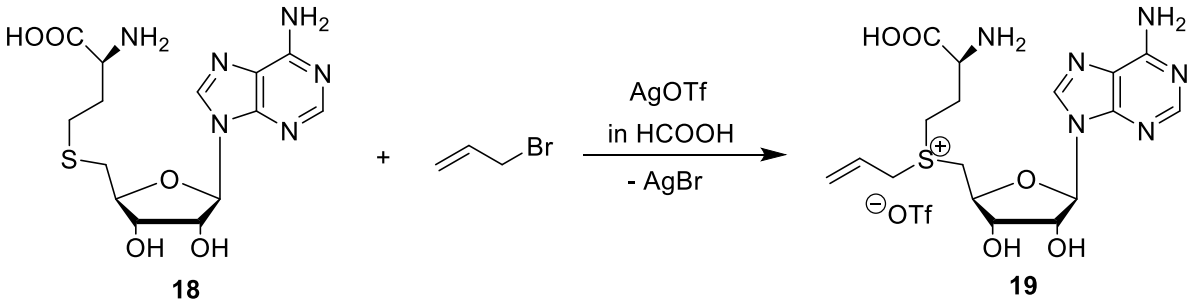


Figure 12: Synthesis of allyl-SAH **19.**

For the alkylation of SAH **18** alkylbromid was used under acidic conditions and catalyzed by AgOTf to synthesize allyl-SAH **19**.[4] The reaction took about 24 h at room temperature. To protect the substrates and products from oxygen the reaction was accomplished under protective gas (N_2).

Synthesis of Allyl-CouBz 18

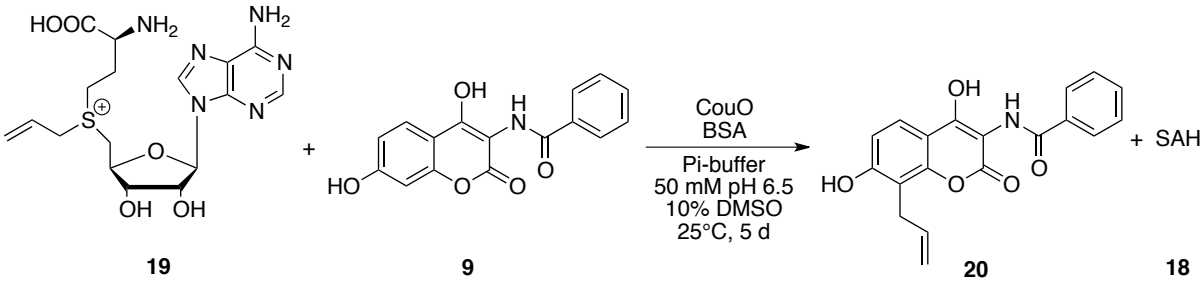


Figure 13: Biocatalytic synthesis of *N*-(8-allyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide **20**.

The transfer of the alkyl-group on CouBz **9** was done biocatalytically using CouO (WT A) at pH 6.5 (optimal pH for CouO 7).[34] The pH value was chosen to reduce the deprotonation degree of the hydroxyl group on the C-7 position of the coumarin ring in order to avoid and/or prevent a possible ring closure with the allyl moiety. The reaction was monitored by HPLC-MS and after five days a conversion of 83% was detected (Figure 14). The reaction was stopped by using 3 N HCl.

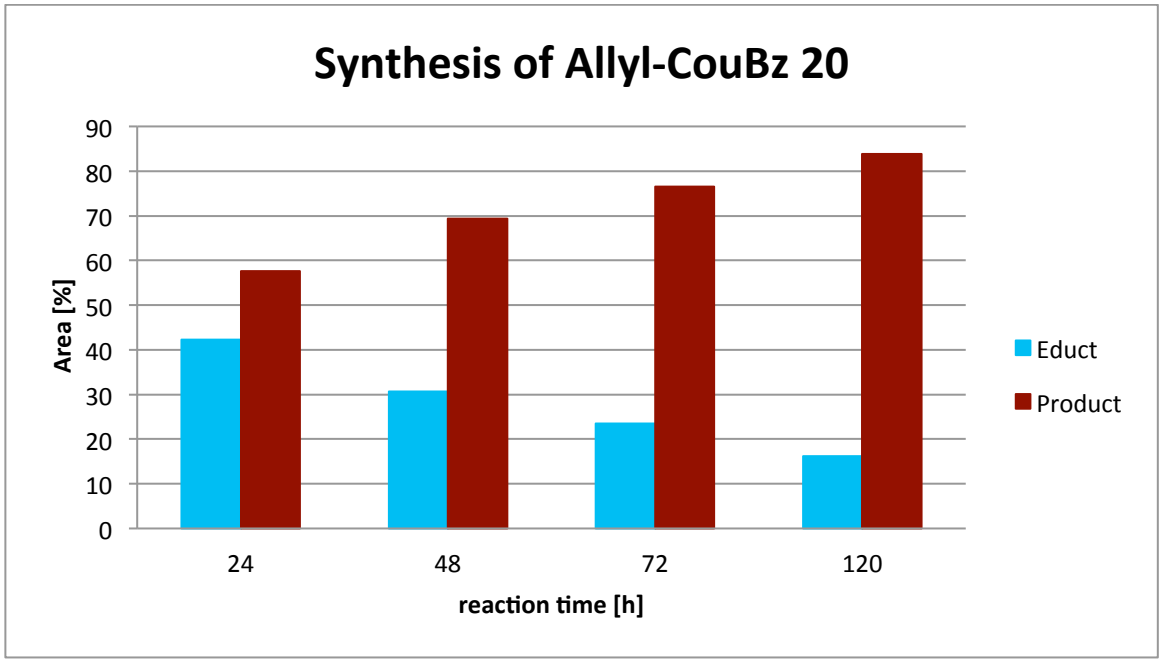
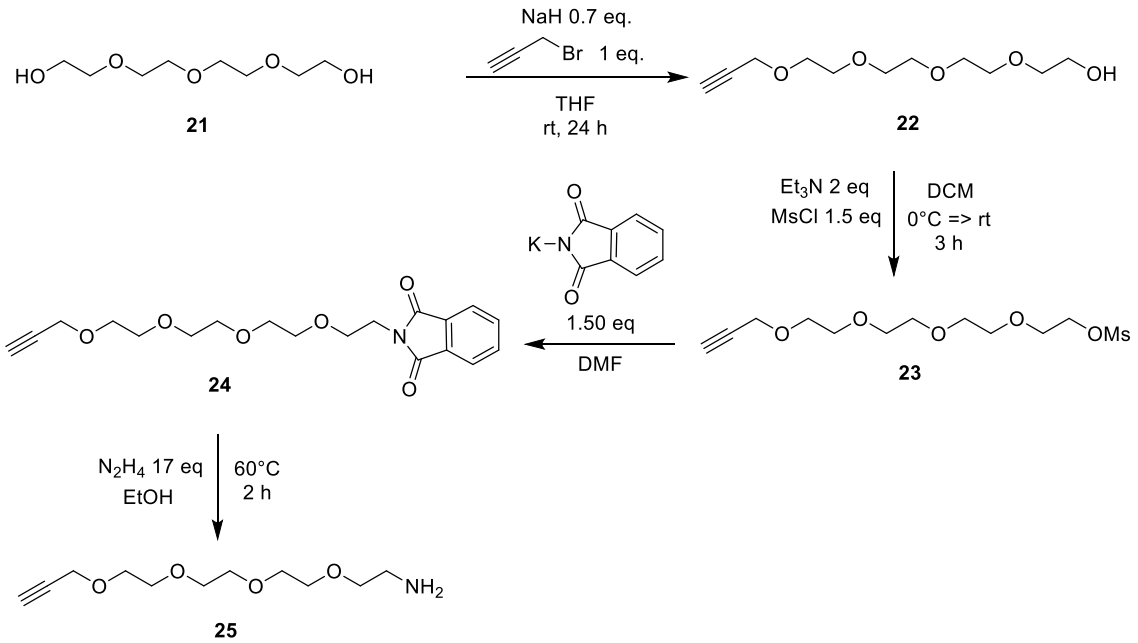


Figure 14: Kinetics of the Allyl-CouBz **20** formation analyzed by HPLC-MS.

4.5. Immobilization of SAH



Scheme 5: Synthesis of the SAM PEG linker.[26]

Compound **25** was prepared as a linking unit for SAH to a solid support in order to immobilize the cofactor SAM.

First the oligo(ethylene glycol) **21** was converted to **22** with propargyl bromide. One equivalent of propargyl bromide was used to obtain the heterobifunctional compound **22**. The heterobifunctionality is needed for the following steps. NMR analysis confirmed the product.[26]

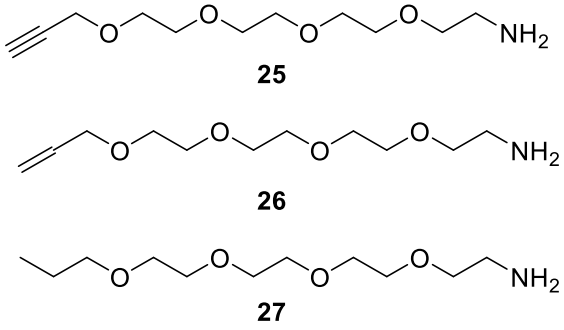
The remaining OH group of the product **22** was converted to a mesylate (-OMs) group. The conversion was complete within a short time (3h).[26]

The next step is the introduction of an amino functionality by performing a Gabriel reaction. The substrate **23** was converted by potassium phthalimide to compound **24**.[26] The conversion was successful but purification had to be done in order to remove the excess of potassium phthalimide.[26] A big drawback by using DMF as solvent is its removal due to its high boiling point (~153°C).

The last step for the synthesis of the linker was the removal of the phthalimide moiety of **24**. This was obtained by using hydrazine (N_2H_4) as a reducing agent.[26] In the first trial 3 different products were detected with HPLC-MS (Figure **15**). The reduced

forms of the alkyne **25**, alkene **26** and alkane **27**, respectively were detectable in the chromatogram.

It was assumed that N_2H_4 is reacting with O_2 to diimide and thus obtaining a reducing effect. Thereby the repetition of this reaction was carried out under different conditions. N_2H_4 got degassed in an ultrasonic bath and the reaction run under protective gas (N_2). The analysis by NMR just showed the alkyne product 3,6,9,12-tetraoxapentadec-14-yn-1-amine **25**.



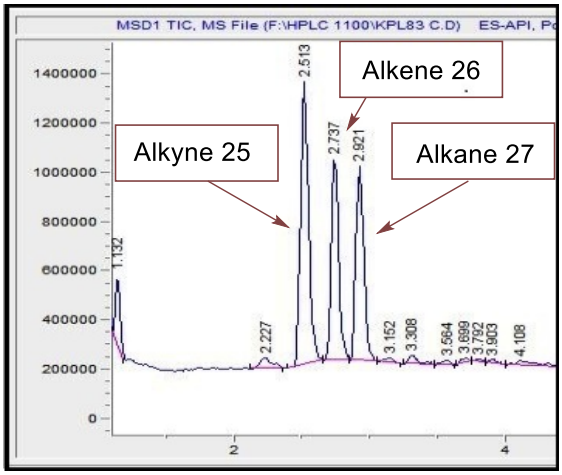


Figure 15: HPLC-MS analysis (retention times shown) of the 3 built products alkyne **25**, alkene **26** and alkane **27**.

Studies for the immobilization of SAH

For the immobilization of SAH two pre-studies have been performed. One transformation was performed with **28** and the other with 6-chloropurine riboside **30** as educt.

The reaction conditions were the same for both syntheses. The difference is the position of the linkage. As already mentioned in the theoretical part the position 4 is the preferred one, but we also performed the linkage on position 3.

Linkage at position 4

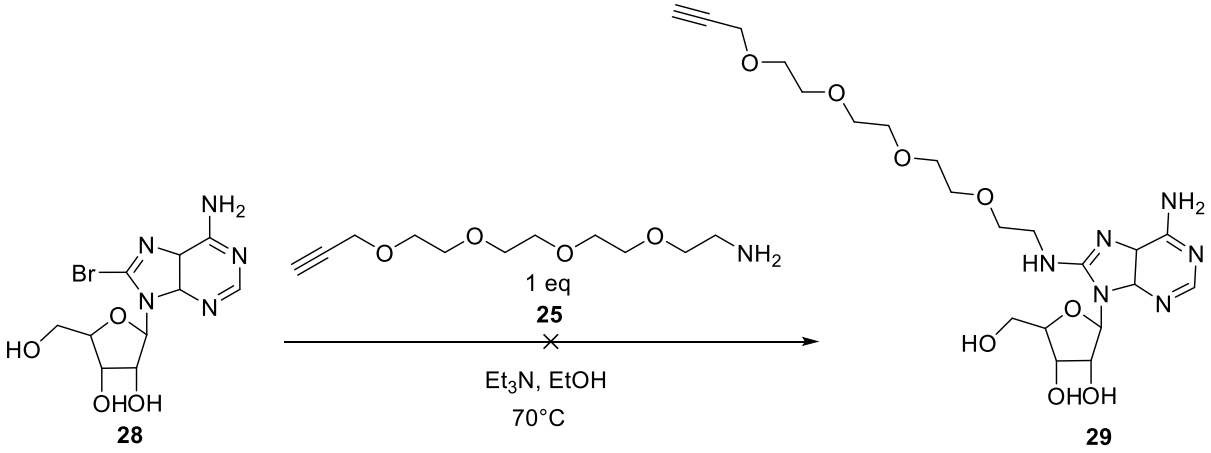


Figure 16: SAH linkage at C-8.

As already mentioned the solubility is a big issue. The educt **28** was not completely soluble despite adding some DMF. Neither 24 h after starting the reaction nor after adding I_2 to achieve an exchange with Br for better conditions for the exchange reaction product was detected in HPLC-MS. The steric hindrance of the attacking PEG-linker might be the reason for the failure.

Linkage at position 3

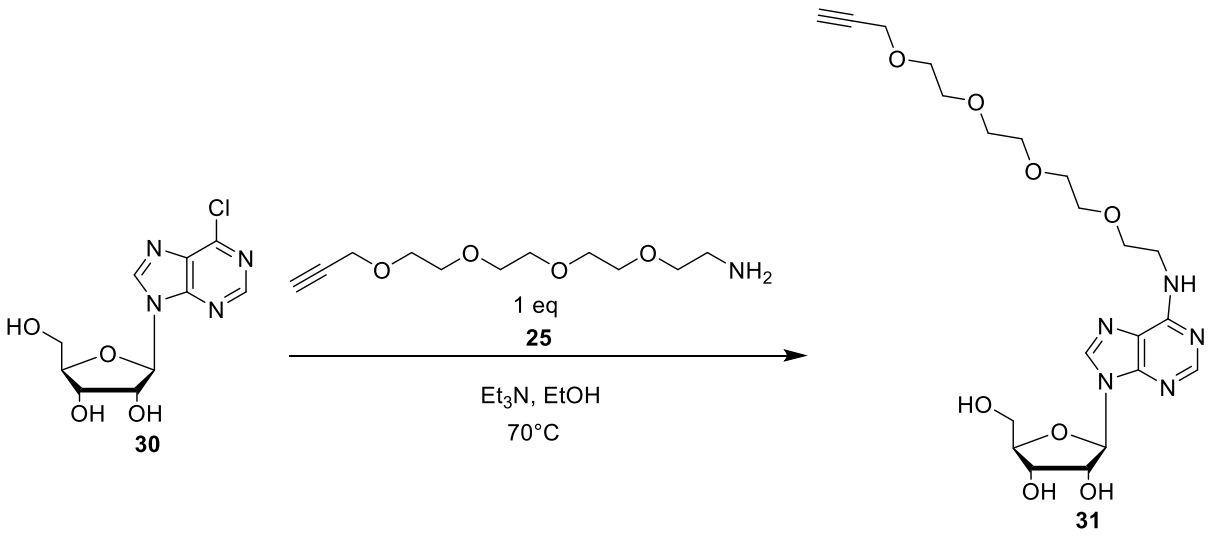


Figure 17: SAH linkage at N-6.

Synthesis of **31** was successful. Performing the same reaction conditions as the reaction before the linkage was achieved. It was possible to link the heterobifunctionalised linker **25** to 6-chloropurine riboside **30** to obtain **31** as was confirmed by HPLC-MS analysis (Figure 17).

5. Conclusion and Outlook

The aminocoumarin CouBz **9** was successfully synthesized on 2.6 mmol scale after developing a new and shorter synthesis pathway. Also the synthesis of the SAM analogue allyl-SAH **19** has been successfully carried out as well as the biocatalytic synthesis of the allyl-CouBz **20**.

The second part of this work involved the synthesis of a linker for the immobilization of SAM. The synthesis of the linker 3,6,9,12-tetraoxapentadec-14-yn-1-amine **25** was successful. The primarily studies with 6-chloropurine riboside **28** are promising.

The next step could be the immobilization of **31** to a solid support leading to immobilized SAM.

The immobilization on different positions of SAM would also be of great importance, because the functional groups are mainly necessary for binding to the active site of the enzyme. For instance at position 3, which was already discussed in the Results and Discussion part.

6. Materials and Methods

6.1. General

By utilization of dry solvents, they were applied as dried solvents and optionally decanted into reagent bottles over a molecular sieve.

When a degassed solvent was required they were degassed as follows: by means of vacuum the solvent was brought to boil in the reaction vessel and then the reaction vessel was purged with inert gas (nitrogen or argon). This operation was repeated at least three times.

Partly the syntheses were performed in flame-dried Schlenk flasks or round-bottom flasks under exclusion of oxygen and water. The Schlenk vessels were closed evacuated in oil pump vacuum, heated and when the vessels were cooled ventilated with inert gas. The respective addition of chemicals was done only by an inert gas back-flow, so that inhibitation by oxygen cannot occur under any circumstances. Hydrogenations were carried out using hydrogen from an Orsatblase instead of using nitrogen as the inert gas. For hydrogenation reactions special safety conditions were necessary. During workup the catalyst filtration was carried out via an inverse filter funnel within a pad of Celite® under inert gas and the catalyst was stored under aqueous conditions.

6.2. Solvents

If dry solvents were needed solvents were used which were stored over 4 Å molecular sieves. The molecular sieves were heated at 200°C in a heating mantle under high vacuum in a 500 ml round-bottom flask for approximately 2 days prior to addition to solvents. The solvent was stored at room temperature under argon atmosphere.

Tetrahydrofuran dry: THF was dried over sodium under argon atmosphere at reflux temperatures until the indicator benzophenone showed a deep blue color. Dried THF was stored over 4 Å molecular sieves under argon.

For reactions which could be performed without inert conditions, as well as for workup and further purification procedures the following solvents listed below were used:

Acetonitrile (AcCN) was purchased from Fluka (34967, with a minimum content of ≥99.9%) in a 2.5 l brown glass bottle and used without further purification.

Cyclohexane (CH) with a minimum content of 99.99% was purchased in 5 l plastic bottles and used without further purification (Fischer Chemical).

Dichloromethane (DCM) was purchased with a minimum content of 99.99% in 5 l plastic bottles and used without further purification.

Diethylether (Et_2O) was commercially available. It was distilled to remove the stabilizer BHT (2,6-di-tert-butyl-4-methylphenole) and stored over KOH in a brown glass bottle.

***N,N*-Dimethylformamid (DMF)** was purchased from Roth (6251.1, with a minimum content of >99.8%) in a 1 l brown glass bottle and used without further purification.

Dimethylsulfoxid (DMSO) was purchased from Acros Organics (127790010, with a minimum content of >99.7%) in a 1 l brown glass bottle and used without further purification.

Ethanol (EtOH) was purchased in 1 l plastic bottles and used without further purification (VWR Chemicals 84105.460).

Ethylacetate (EE) with a minimum content of 99.97% was purchased in 5 l plastic bottles and used without further purification.

Methanol (MeOH) with a minimum content of 99.99% was purchased in 5 l plastic bottles and used without further purification (VWR Chemicals 20847.360).

Pyridine (Py) was purchased from Merck (822301, with a minimum content of 99%) in a 2.5 l brown glass bottle and used without further purification.

Sodiumbicarbonate ($NaHCO_3$) was added to distilled water until saturation was obtained (~10 g per 100 mL).

Tetrahydrofuran (THF) was purchased from Merck (985701, with a minimum content of 99.7%) in a 2.5 l brown glass bottle and used without further purification.

Triethylamine (Et_3N) was purchased from Sigma-Aldrich (T0886, with a minimum content of 99%) in a 1 l brown glass bottle and used without further purification.

6.3. Reagents

The chemicals used were purchased from the following companies: Sigma Aldrich, Acros Organics, Fluka and Alfa Aesar. The reagents were used without any further purification steps.

6.4. Analytical methods

Thin layer chromatography

TLC plates from Merck (DC aluminum foil, silica gel 60 F254, 20 x 20 cm) were used. The sample was applied using a capillary glas to the plate and detected with a UV lamp at a wavelength of 366 nm or 254 nm or with different staining solutions such as CAM (cer ammonium molybdate), ninhydrin or $KMnO_4$ by additional treatment with hot-air flow.

Staining solutions:

- **CAM:** 2.0 g cer(IV)-sulfate, 50.0 g ammonium molybdate and 50 ml conc. H_2SO_4 were added to 400 ml distilled water.
- **Ninhydrin:** 250.0 mg ninhydrin, 5 ml pyridine were added to 95 ml MeOH.
- **$KMnO_4$:** 18 g $KMnO_4$, 120 g K_2CO_3 and 30 ml 5% NaOH were added to 1800 ml dest. H_2O.

Flash Chromatography

For preparative column chromatography, the silica gel 60 Å of Acros Organics with a grain size between 35 µm and 70 µm was used. In this method, the products were pressed with pressure through the column. It depended on product quantity and/or product properties which column lengths and amount of silica gel, which were used. The R_f-values of the desired compound in the elution solvent was set to be around 0.20. The approximately sized columns and applied solvent compositions are given in the experimental procedures. For optimal separation of the compounds the overall length of the SiO_2 pad was between 15 and 30 cm. Typically the mass of used silica was between 50 to 100 times (w/w) of the mass of the crude product. There were various eluents used (dichloromethane/acetone, cyclohexane/ethyl acetate) and solvent gradients.

Analytic High Performance Liquid Chromatography

For HPLC analysis an Agilent 1200 system was used consisting of the vacuum degasser G1379B, the binary pump G1312B, the autosampler G 1367C and the column compartment G1316B. Detection was done by the multi wavelength detector (MWD) G1365C and a quadrupol-MS6120 with an electrospray ionization (ESI) unit. As stationary phase two different columns with reversed phase were used.

HPLC columns:

Macherey Nagel Nucleodur® C_{18} ec 150 x 4 mm, 5 µm

Agilent Poroshell® C_{18} 100 x 3 mm, 2.7 µm

Methods:

"6PurinePEG _095ml_min"

A: 0.01% HCOOH in H_2O, B: AcCN

Gradient: 0 – 0.1 min 2% B, 0.1 - 8 min to 100% B, 8 – 10.1 min at 100% B, 10.1 – 12 min to 2% B.

40°C, flow: 0.95 ml/min, overall time: 12 min, ESI^+, UV: 220, 254, 260 nm, Poroshell

"CouBz_NO_Felix_095ML_MIN"

A: 0.01% HCOOH in H_2O, B: AcCN

Gradient: 0 – 0.1 min 2% B, 0.1 – 8 min to 100% B, 8 – 10.1 min at 100% B, 10.1 – 12 min to 2% B.

40°C, flow: 0.95 ml/min, overall time: 12 min, ESI^+, UV: 220, 254, 305 nm, Nucleodur

High Performance Liquid Chromatography

For reaction monitoring "Shimdazu HPLC MS" Nexera system was used. The system consists of the Nexera Pump LC-39 AD and the 6-way valve FCV-20AH_2. The column is placed in the Prominence Column oven CTO-20A. For the determination of the mass, the mass detector LCMS-2020 was used, which consists of an ESI-ionisation and a quadrupole-analyser. The detection was done by the Prominence

Diodenarray detector SPD-M20A. The separation was performed with two different columns with reversed phase.

HPLC columns:

Agilent Poroshell® C_{18} 100 x 3 mm, 2.7 µm

Methods:

„CouBz_NH4OAc_Poroshell_7zu65_short“

A: 10 mM NH_4OAc pH 5.5, B: AcCN

Gradient: 0 – 2 min to 7% B, 2 – 5 min to 62.8% B, 5 – 5.5 min at 62.8% B, 5.5 – 5.6 min to 2% B.

40°C, flow: 1 ml/min, overall time: 6.4 min

„CouBz_NH4OAc_Poroshell_7zu100_short“

„CouPhe_NH4OAc_Poroshell_7zu100_short”

A: 10 mM NH_4OAc pH 5.5, B: AcCN

Gradient: 0 – 2 min to 7% B, 2 – 7 min to 100% B, 7 – 8 min at 100% B, 8 – 9 min to 7% B

40°C, flow: 1 ml/min, overall time: 10 min

„Cou_2OH_NH4OAc_Poroshell_2zu8zu100_short”

A: 10 mM NH_4OAc pH 5.5, B: AcCN

Gradient: 0 – 2 min to 7% B, 2 – 7 min to 80% B, 7 – 8 min at 100% B, 8 – 9 min to 2% B

40°C, flow: 1 ml/min, overall time: 10 min

“CoumarinNH2_ACN_H2O_2-100”

A: 0.01% HCOOH in H_2O, B: AcCN

Gradient: 0.1 – 8 min to 100% B, 8 – 10 min at 100% B, 10 – 10.1 min to 2% B

40°C, flow: 0.7 ml/min, ESI^+, overall time: 12 min

„CouBz_NH4OAc_ACN10-90”

A: 0.01% HCOOH in H_2O, B: AcCN 90/10, 40°C, flow: 1 ml/min, UV: 230, 254, 260, 305 nm, Poroshell, overall time: 15 min

Semipreparative High Performance Liquid Chromatography

For preparative analysis the HPLC "UltiMate 3000" was used. The system consists of the Dionex UltiMate 3000 Autosampler and the Dionex UltiMate 3000 Pump. The column is placed in the Dionex UltiMate 3000 Column Compartment. As detector the Dionex UltiMate 3000 Diode Array Detector is used and for fractioning the Dionex UltiMate 3000 Automated Fraction Collector.

Column:

Macherey Nagel Nucleodur® C_{18} ec 125 x 21 mm, 5 µm

Method:

„KPL_NucleodurC18_001HCOOH_30to70"

A: 0.01% HCOOH in H_2O, B: AcCN, 30°C, flow: 12 ml/min, UV: 316, 260 nm
Gradient: 0 – 6 min to 30% B, 6 – 12 min to 70% B, 12 – 14 min at 70% B, 14 – 16 min to 30% B.

„KPL_NucleodurC18_NH4OAc_2to65v2"

A: 10 mM NH_4OAc pH 6.5, B: AcCN, 30°C, flow: 10 ml/min, UV: 210, 260, 305 nm
Gradient: 0 – 4 min to 7% B, 4 – 10 min to 65% B, 10 – 14 min at 65% B, 14 – 18 min to 95% B, 20 – 20.5 min to 2% B, 20.5 – 25 min at 2% B.

Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR-spectra were recorded with a Bruker AVANCE III spectrometer which was equipped with an autosampler (300.36 MHz-^{1}H-NMR, 75.53 MHz-^{13}C-NMR) or with a Varian Inova-500 (500MHz) spectrometer.

The ^{1}H, ^{13}C and ATP chemical shifts were measured relative to the rest proton signal of the deuterated solvents as internal reference. To facilitate the interpretation, the ^{13}C-spectra were proton decoupled to gain better identification of the peaks. APT spectra are recorded for differentiation of C-atoms if necessary. For the explicit identification of certain compounds also two-dimensional spectra such as ^{1}H^{1}H-COSY, ^{1}H^{13}C-HSQC and ^{1}H^{13}C-HMBC were recorded.

The respective signals are as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet) and dd (double of doublet) indicated. Quaternary carbons are figured as C_q and arylic carbon atoms as CH_{Ar}. Chemical shifts δ are expressed in parts per million (ppm), coupling constants *J* are indicated in Hz (Hertz), integral and structural fragments are respectively given. As solvents deuterated chloroform ($CDCl_3$) or deuterated DMSO (DMSO-d_6) were used

6.5. Biocatalysis

CouO

The C-Methyltransferase CouO crude lysates were used.

Two different CouO crude lysates were screened for activity, WT N (wild type new prepared 2014) and WT A (wild type alt prepared 2009).

Enzyme screening assay conditions

Enzyme preparation	crude lysate CouO WT A (2009) in buffer
	crude lysate CouO WT N (2014) in buffer
Substrate	1 mM
SAM	2 mM
DMSO	10% v/v
Bovine serum albumin	1 mg/ml
Buffer	50 mM NaPi-buffer pH 7

Stock solutions used:

Substrate 10 mM in DMSO

SAM 20 mM and BSA 2 mg/ml in buffer

Crude lysates of CouO were incubated in 0.1 ml scale in a thermomixer at 30 °C and 1000 rpm (revolutions per minute) for 24 hours containing 1 mM substrate, 2 mM SAM and 1 mg/ml BSA in NaPi-buffer in 10% DMSO. The amount of lysates was set

to 80% of total reaction volume. The assay was stopped by heating the vials for 10 min at 80 °C and 1000 rpm. Subsequently the mixtures were centrifugated at 13.000 rpm for 10 min. The supernatant was filtered through 3kDa-cut-off test tubes and were again centrifugated for 10 min. The filtrate was analyzed with HPLC without further dilution.

Preparative scale experiments

General procedure for the preparation of the SAM analogs for preparative scale experiments:

100 mg SAH **16** (A9384) (260 µmol, 1 eq) was solved in 5 ml formic acid. 200 mg fine ground AgOTf (780 µmol, 3 eq) was added. The mixture was cooled to 0°C for 10 min before adding 2.3 ml allyl bromide (26 mmol, 100 eq). After few minutes precipitation occurred. The suspension was stirred at room temperature for 24 h. After complete conversion the reaction mixture was diluted with water (15 ml) and filtered through cellulose filter to remove AgOTf and AgBr. The reaction mixture was extracted 3 times with Et_2O (3 x 15 ml) in order to remove excess Allylbromide. The aqueous solution was lyophilized to dryness and used without purification for the synthesis of allyl-CouBz. The reaction was monitored by HPLC.

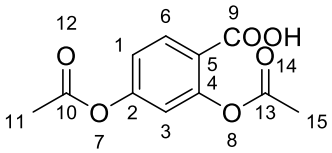
General procedure for the preparative scale experiments with SAM analogue.

A mixture of 25 mg CouBz (84 µmol, 1 eq), 71.6 mg allyl-SAH (168 µmol, 2 eq) and 25 mg BSA were solved in 25 ml crude enzyme lysates, 2.5 ml 50 mM NaPi-buffer pH 6.5 and 2.5 ml DMSO at room temperature. The mixture was shaken at 25°C and 120 rpm for 24 h. Then the first reaction control was done by Shimadzu-HPLC. The conversion was around 50%. After 5 d the enzyme was denaturated by adding 3 N HCl and shaking for 10. The mixture was then centrifuged at 4000 g for 20 min. The supernatant was extracted 3 times with EE. The precipitate was suspended in EE, thoroughly vortexed and centrifuged for 10 min at 4000 g. The combined organic phases were dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. To purify the product a preparative HPLC was done. The product was characterized by HPLC-MS and NMR analysis.

7. Experimental part

7.1. Synthesis I of CouBz

2,4-Diacetoxybenzoic acid (2)

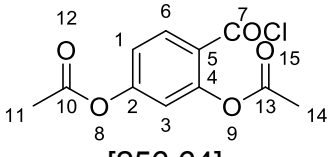


[138.19]

$C_{11}H_{10}O_6$

In 30 ml pyridine 5.0 g 2,4-dihydroxybenzoic acid **1** (32 mmol, 1 eq) were solved in a light protected round-bottom flask. After that 15.3 ml acetic anhydride (162 mmol, 5 eq) and 0.4 g DMAP (6.6 mmol, 0.1 eq) were added. The reaction mixture was stirred at r.t. overnight. The orange red reaction solution changed its color to yellow after acidifying with 3 N HCl (10 ml) at 0°C to pH 1. The reaction was extracted three times with EE and the combined organic phases were washed with brine. The organic phase was dried over Na_2SO_4 and most of the solvent was removed to allow crystallization from the mother lye. The resulting crystals were collected and dried. The resulting white crystals were dried under vacuum to give 5.2 g (67 %) yield. ^{1}H NMR (300 MHz, DMSO-d*6*) δ ppm: 13.13 (br, 1H, H-9), 7.98 (d, *J* = 8.6 Hz, 1H, H-6), 7.18 (dd, J_1 = 8.6, J_2 = 2.1 Hz, 1H, H-3), 7.09 (d, *J* = 2.0 Hz, 1H, H-1), 2.29 (s, 3H, H-15), 2.25 (s, 3H, H-11). ^{13}C (76 MHz) δ ppm: 168.98 (C_q, C-13) 168.62 (C_q, C-10), 164.98 (C_q, C-9), 154.01 (C_q, C-2), 151.04 (C_q, C-4), 132.47 (CH_{ar}, C-6), 121.60 (C_q, C-5), 119.67 (CH_{ar}, C-1), 117.63 (CH_{ar}, C-3).

4-(Chlorocarbonyl)-1,3-phenylene diacetate (3)

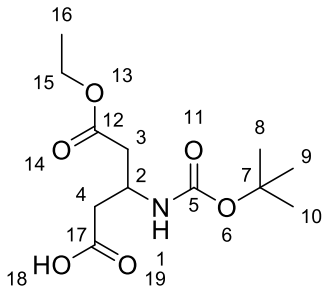


[256.64]

$C_{11}H_9ClO_5$

Solving 1.0 g 2,4-diacetoxybenzoic acid **2** (4.2 mmol, 1 eq) in 10 ml DCM. After everything was solved 6.1 ml $SOCl_2$ (10 g, 84 mmol, 20 eq) was added. The mixture was heated to reflux overnight. The next step was to distill the reaction mixture to remove DCM and $SOCl_2$. Without any further purification the crude product was used for the next step.

2-((*tert*-Butoxycarbonyl)amino)-3-ethoxy-3-oxopropanoic acid (5)

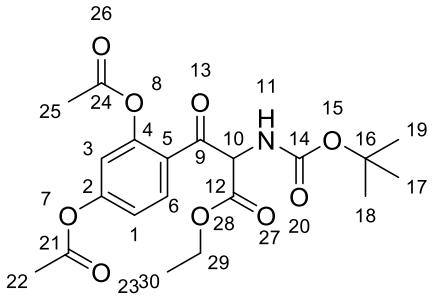


[247.25]

$C_{10}H_{17}NO_6$

To a white suspension of 2.3 g KOH (40 mmol, 1 eq) solved in 50 ml EtOH 10 ml diethyl(boc-amino)malonate **4** (11 g, 40 mmol, 1 eq) was added. The reaction mixture changed its color from white into yellow. After three hours 90 % of the solvent was removed and aq. $NaHCO_3$ was added to pH 11. The mixture was extracted three times with EE to remove the educt. Then the aqueous phase was cooled to 0°C and acidified with $KHSO_4$ to pH 3 and extracted three times with EE. The combined organic phases were washed once with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure (bath temperature 20°C) and the resulting oil was dried under vacuum. At the end 6.9 g (70 %) white powder resulted. ^{1}H NMR (300 MHz, DMSO) δ ppm: 13.37 (s, 1H,H-18), 4.71 (d, *J* = 8.0 Hz, 1H, H-2), 4.11-4.18 (m, *J* = 6.9 Hz, 2H, H-15), 1.38 (s, 9H, H-8, H-9, H-10), 1.19 (t, *J* = 7.1 Hz, 3H, H-16). ^{13}C (76 MHz) δ ppm: 167.72 (C_q, C-17), 167.20 (C_q, C-12), 155.05 (C_q, C-5), 78.90 (C_q, C-7), 61.28 (CH, C-2), 57.55 (CH_2, C-15), 28.06 (CH_3, C-8, C-9, C-10), 13.89 (CH_3, C-16).

4-(2-((*tert*-Butoxycarbonyl)amino)-3-ethoxy-3-oxopropanoyl)-1,3-phenylene diacetate (6)

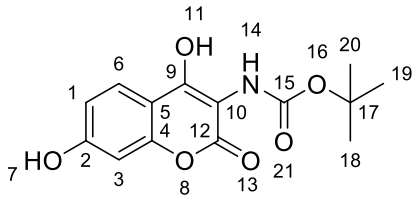


[423.42]

$C_{20}H_{25}NO_9$

Solving 1.5 g 2-(*tert*-butoxycarbonylamino)-3-ethoxy-3-oxopropanoic acid **5** (5.9 mmol, 1.4 eq) completely in 15 ml dry THF. Adding 3.7 ml Et_3N (2.7 g, 27 mmol, 6.3 eq) and 1.4 g $MgCl_2$ anhydride (14 mmol, 3.4 eq) at 0°C. After two hours adding 1.1 g 4-(chlorocarbonyl)-1,3-phenylene diacetate **3** (4.2 mmol, 1 eq), which was solved in 25 ml dry THF to the reaction mixture at 0°C. The milky yellow-orange mixture was stirred overnight at room temperature. The reaction was quenched with sat. NH_4Cl until the reaction solution changed its color into clear dark-red. The reaction was extracted three times with EE and the combined organic phases were dried over Na_2SO_4. After removing the solvent 1.5 g (84 %) brown oil resulted. The crude product was used for the next step without purification.

***tert*-Butyl (4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)carbamate (7)**



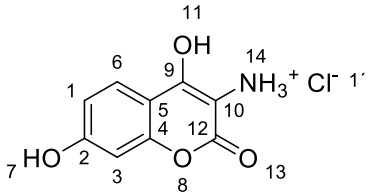
[293.27]

$C_{14}H_{15}NO_6$

Solving 1.5 g crude 4-(2-(*tert*-butoxycarbonylamino)-3-ethoxy-3-oxopropanoyl)-1,3,-phenyl-diacetate **6** (3.5 mmol, 1 eq) in 8.5 ml MeOH and slowly adding 11 ml 1.5 N NaOH. After three hours stirring at room temperature the reaction was acidified with 1 N HCl to pH 3. The yellow-

orange solution was extracted three times with EE and washed with brine. The combined organic phases were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude orange-brown oil was purified by column chromatography. The pure product resulted in 100 mg (10 %) white solid. ^{1}H NMR (300 MHz, DMSO) δ ppm: 10.71 (s, 1H,H-7), 10.45 (s, 1H, H-14), 7.64 (d, J = 8.7 Hz, 1H, H-6), 6.37 (dd, J_1 = 8.7, J_2 = 2.1 Hz, 1H, H-1), 6.29 (d, J = 2.1 Hz, 1H, H-3), 1.38 (d, J = 8.8 Hz, 9H, H-18, H-19, H-20). ^{13}C NMR (76 MHz, DMSO) δ ppm: 169.55 (C_q, C-9), 164.20 (C_q, C-12), 162.66 (C_q, C-2), 131.58 (CH_{ar}, C-6), 108.34 (CH_{ar}, C-1), 103.94 (C_q, C-5), 102.46 (CH_{ar}, C-3), 85.92 (C_q, C-10), 77.0 (C_q, C-17), 28.19 (CH_3, C-18, C-19, C-20).

4,7-Dihydroxy-2-oxo-2*H*-chromen-3-aminium chloride (8)

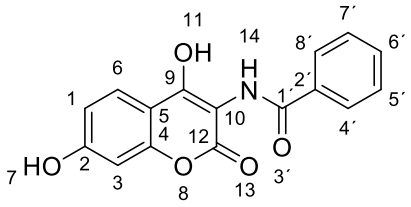


[229.62]

$C_9H_8ClNO_4$

A mixture of 10 ml Et_2O, 2 ml MeOH and 1.7 ml acetyl chloride was prepared and cooled to 0°C. Into a mixture of 10 ml Et_2O, 2 ml MeOH and 0.5 ml 1 N HCl 130 mg *tert*-butyl (4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)carbamate **7** (0.4 mmol, 1 eq) was added and a yellowish solution appeared. The cooled mixture was added. After 24 h the reaction mixture was neutralized by Na_2HCO_3 and extracted three times with EE. The combined organic phases were washed with H_2O. The organic phase was dried over Na_2SO_4 and the solvent was removed. At the end 141.7 mg (quantitative) yellow solid resulted. ^{1}H NMR (300 MHz, DMSO) δ ppm: 10.71 (s, 1H, H-11), 10.46 (s, 1H, H-7), 7.63 (d, J = 8.7 Hz, 1H, C-6), 6.37 (dd, J_1 = 8.7, J_2 = 2.2 Hz, 1H, C-1), 6.29 (d, J = 2.2 Hz, 1H, H-3). ^{13}C NMR (76 MHz, DMSO) δ ppm: 169.56 (C_q, C-12), 164.22 (C_q, C-2), 162.68 (C_q, C-4), 131.61 (CH_{ar}, C-6), 108.36 (CH_{ar}, C-1), 103.96 (C_q, C-5, C-10), 102.48 (C_q, C-3).

***N*-(4,7-Dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide (9)**



[297.27]

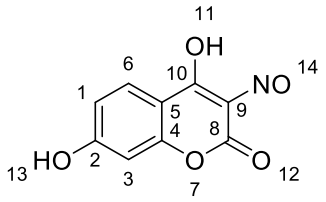
$C_{16}H_{11}NO_6$

The last step of the synthesis was to solve 893 mg 4,7-dihydroxy-2-oxo-2*H*-chromen-3-aminium chloride **8** (3.9 mmol, 1 eq) completely in 50 ml EE. Adding 2.3 ml benzoyl chloride (19.5 mmol, 5 eq) and 5.4 ml Et_3N (39 mmol, 10 eq). Stirring the milky orange reaction mixture at r.t. overnight. Filtering the reaction mixture and removing the solvent of the filtrate under reduced pressure. Adding 40 ml 1.5 N NaOH and 40 ml MeOH to the brown oil and stirring at r.t. overnight. Adding 10% HCl to achieve pH 3, extracting 3 times with EE and washing ones the combined organic phases with brine. Drying the organic phase over Na_2SO_4 and removing the solvent under reduced pressure. To purify the brown oil a column chromatography was done. After drying the product under vacuum 137 mg (12%) brown oil resulted.

HPLC-MS [*"CouBz_NH4OAc_ACN10-90"*]: t_R = 2.007 min, $[MH^+]$ = 298.

7.2. Synthesis II of CouBz

4,7-Dihydroxy-3-nitroso-2*H*-chromen-2-one (14)

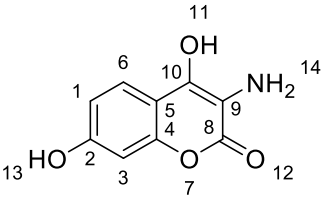


[207.14]

$C_9H_5NO_5$

Solving 250 mg 4,7-dihydroxy-2*H*-chromen-2-one **10** (1.4 mmol, 1 eq) in 20 ml AcOH. Slowly adding 197 µl *t*BuONO (1.4 mmol, 1 eq) to the reaction mixture and its color changed from white into a yellow solution. Stirring the reaction mixture for 4 h at r.t. Afterwards, the crude product was used for the next step of the one-pot synthesis without purification.

3-Amino-4,7-dihydroxy-2*H*-chromen-2-one (15)



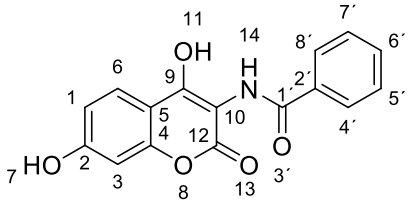
[193.16]

$C_9H_7NO_4$

The reduction was done by adding 20 ml MeOH under H_2-atmosphere with 50 mg 10% Pd/C to the reaction mixture of 4,7-dihydroxy-3-nitroso-2*H*-chromen-2-one **14**. The black reaction mixture was stirred at r.t. overnight. The Pd/C was filtered off and the solvent was removed under reduced pressure. The resulting brown solid was dried over vacuum to give 495 mg (quantitative) brown solid. The product was used for the next step without purification.

HPLC-MS [*"CoumarinNH2_ACN_H2O_2-100"*]: t_R = 3.233 min, [MS^+] = 194.

***N*-(4,7-Dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide (9)**



[297.27]

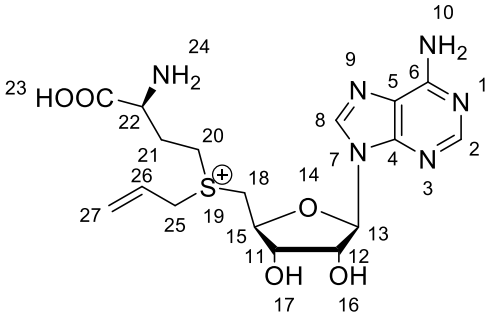
$C_{16}H_{11}NO_6$

Solving 500 mg 3-amino-4,7-dihydroxy-2*H*-chromen-2-one **15** (2.6 mmol, 1 eq) in 10 ml abs. DCM. Adding 3.6 ml Et_3N (25.9 mmol, 10 eq), 1.5 ml benzoyl chloride (12.9 mmol, 5 eq) and 4.7 g DMAP (38.8 mmol, 15 eq). Stirring the reaction mixture at room temperature overnight. After TC control (CH/EE/AcOH 2:4:1) the brown reaction mixture was filtered and the solvent of the reaction solution was removed. After that 25 ml MeOH and 25 ml 3 N NaOH was added. Stirring the brown reaction mixture 48 h at r.t. After TC control (CH/EE/AcOH 10:5:1, UV and CAM) the reaction was quenched with 10% HCl and the brown solution changed into a clear red solution. Extracting the reaction mixture three times with EE and ones with brine. Drying the organic phase with Na_2SO_4 and removing the solvent. Column chromatography was done to purify the crude product. At the end 600 mg (78 %) of a brown solid resulted. ^{1}H NMR (300 MHz, DMSO) δ ppm: 10.59 (s, 1H, H-7), 9.37 (s, 1H, H-14), 8.00 (s, 2H, C-2´, C-4´), 7.69 (d, *J* = 8.6 Hz, 1H, H-6), 7.53 (d, *J* = 7.1 Hz, 1H, C-6´), 7.48 (d, *J* = 7.4 Hz, 2H, H-5´, H-7´), 6.80 (d, *J* = 8.7 Hz, 1H, H-1), 6.72 (s, 1H, H-3). ^{13}C NMR (76 MHz, DMSO) δ ppm: 167.36 (C_q, C-1´), 166.43 (C_q, C-9), 161.26 (C_q, C-12), 161.04 (C_q, C-2), 153.60 (C_q, C-4), 134.08 (C_q, C-2´), 131.42 (CH_{ar}, C-6´), 128.57 (CH_{ar}, C-6), 128.09 (CH_{ar}, C-5´, C-7´), 127.94 (CH_{ar}, C-4´, C-8´), 112.67 (CH_{ar}, C-1), 108.90 (C_q, C-5), 101.80 (CH_{ar}, C-3), 99.73 (C_q, C-10).

HPLC-MS [*„CouBz_NH4OAc_Poroshell_7zu100_short“*]: t_R = 2.827 min, [MS^+] = 298.

7.3. Synthesis of Allyl-CouBz

Allyl((*S*)-3-amino-3-carboxypropyl)(((2*S*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)sulfonium (19)



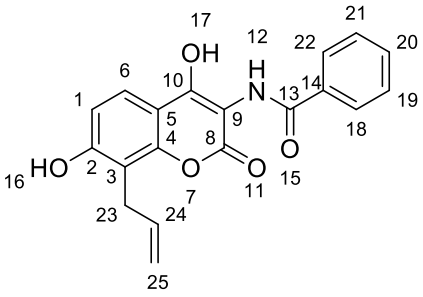
[425.48]

$C_{17}H_{25}N_6O_5S^+$

Experiment description see page 35.

228 mg (206%) yellow oil resulted and was used without purification for the next step.

***N*-(8-Allyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide (20)**



[337.33]

$C_{19}H_{15}NO_5$

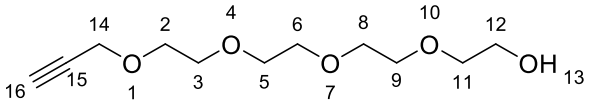
A mixture of 25 mg *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide **9** (84 µmol, 1 eq), 71.6 mg allyl((*S*)-3-amino-3-carboxypropyl)(((2*S*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)sulfonium **17** (168 µmol, 2 eq) and 25 mg BSA were solved in 25 ml crude enzyme lysates, 2.5 ml 50 mM NaPi-buffer pH 6.5 and 2.5 ml DMSO at r.t. The mixture was

shaken at 25°C and 120 rpm for 24 h. Then the first reaction control was done by HPLC-MS. It was still around 50% of the educt visible, therefore the reaction was continued. The enzyme was denaturated after 5 d by adding 5 ml 2 N HCl and shaking at 120 rpm for 20 min. The mixture was than centrifuged at 4000 g for 20 min. The supernatant was extracted 3 times with EE. The precipitate was suspended in EE, thoroughly vortexed and centrifuged for 10 min at 4000 g. The combined organic phases were dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. To purify the product preparative HPLC was used. ^{1}H NMR (300 MHz, DMSO) δ ppm: 7.96 (d, *J* = 6.8 Hz, 2H, H-18, H-22), 7.54 – 7.41 (m, 4H, H-19 H-21, H-6), 6.66 (d, *J* = 8.5 Hz, 1H, H-1), 5.94 (dd, *J* = 16.9, 10.2 Hz, 1H, H-24), 4.94 (t, *J* = 11.6 Hz, 2H, H-25), 3.42 (d, *J* = 5.6 Hz, 2H, H-23). ^{13}C NMR (76 MHz, DMSO) δ ppm: 165.17 (C_q, C-13), 162.39 (C_q, C-10), 156.73 (C_q, C-8), 152.24 (C_q, C-4), 136.43 (CH, C-24), 135.68 (C_q, C-14) 130.45 (CH_{ar}, C-20), 127.90 (CH_{ar}, C-19, C-21), 127.57 (CH_{ar}, C-18, C-22), 123.06 (CH_{ar}, C-6), 115.88 (C_q, C-3), 114.42 (CH_2, C-25), 111.22 (CH_{ar}, C-1), 109.75 (C_q, C-5),96.12 (C_q, C-9), 26.85 (CH_2, C-23).

[*„CouBz_NH4OAc_Poroshell_7zu65_short“*]: t_R = 3.566 min, [MS^+] = 338.

7.4. Immobilization of SAH

3,6,9,12-Tetraoxapentadec-14-yn-1-ol (22)



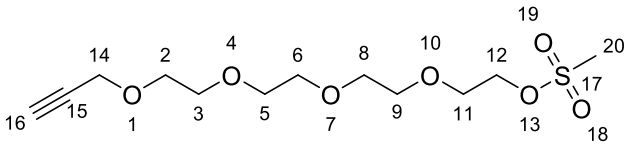
[232.28]

$C_{11}H_{20}O_5$

Into a two-neck round-bottom flask with 75 ml THF 720 mg NaH (18 mmol, 0.7 eq) was added. A solution of 5.0 g tetraethylenglykol **19** (25 mmol, 1 eq) and 100 ml THF was added to the white reaction mixture over 45 min. A solution of 3.1 g propargyl bromide (25 mmol, 1 eq) in 75 ml THF was added over 30 min. The reaction was then heated to 60°C and stirred for 16 h. The first reaction control was done by TLC (DCM/Aceton 9:1, UV and $KMnO_4$).

The reaction was quenched with 200 ml 3% HCl and an orange solution arose. Under reduced pressure the solvent of the organic phase was removed. The crude product was solved in 100 ml DCM and dried over Na_2SO_4. After filtering the Na_2SO_4 the solvent was removed under reduced pressure and the brown oil was purified by a column chromatography. In total 2.6 g (45%) brown oil. ^{1}H NMR (300 MHz, CDCl3) δ ppm: 4.19 (d, *J* = 2.2 Hz, 2H, H-14), 3.67 (d, *J* = 9.1 Hz, 14H, C-2, C-3, C-5, C-6, C-8, C-9, C-11), 2.42 (t, *J* = 2.1 Hz, 1H, H-16). ^{13}C NMR (76 MHz, CDCl3) δ ppm: 79.80 (C_q, C-15), 74.62 (CH, C-16), 70.74 (CH_2, C-5, C-6, C-8, C-9), 70.71 (CH_2, C-11), 70.45 (CH_2, C-3), 69.25 (CH_2, C-2, C-3), 58.52 (CH_2, C-14).

3,6,9,12-Tetraoxapentadec-14-yn-1-ylmethanesulfonate (23)



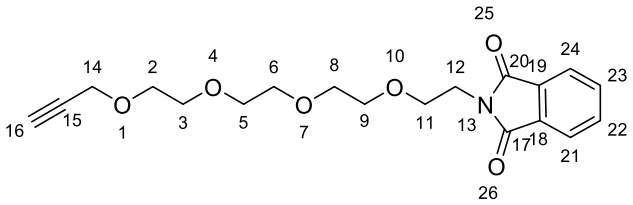
[310.36]

$C_{12}H_{22}O_7S$

In a 250 ml two-neck round-bottom flask 2.7 g 3,6,9,12-tetraoxapentadec-14-yn-1-ol **20** (11 mmol, 1 eq) was solved in 50 ml DCM and 3.2 ml Et_3N (23 mmol, 2 eq) was added at 0°C. Via dropping funnel 1.3 ml MsCl (17 mmol, 1.5 eq) in 40 ml DCM was added to the reaction mixture. The reaction was stirred for 2 h at 0°C and after that stirred at r.t. After 2 h reaction control was done with TLC (5% MeOH in DCM, UV and $KMnO_4$). The reaction mixture was concentrated to dryness and completely re-dissolved in 150 ml DCM and washed with 150 ml 3% HCl that the product was in the organic phase. The organic phase was dried over Na_2SO_4 and the solvent was removed. The crude product was 2.97 g (84%) brown oil. ^{1}H NMR (300 MHz, CDCl3) δ ppm: 4.15 (d, *J* = 2.2 Hz, 2H, H-14), 3.75-3.67 (m, 2H, H-12), 3.63 (s, 1H, H-11), 3.60 (d, *J* = 4.1 Hz, 12H, H-2, H-3, H-5, H-6, H-8, H-9), 3.04 (s, 3H, H-20), 2.41 (d, *J* = 2.5 Hz, 1H, H-16). ^{13}C (76 MHz, $CDCl_3$) δ ppm: 79.68 (C_q, C-15), 74.60 (CH, C-16), 70.58 (CH_2, C-5, C-6, C-8, C-9), 70.38 (CH_2, C-3), 69.37

(CH_2, C-2), 69.10 (CH_2, C-11), 68.99 (CH_2, C-12), 58.36 (CH_2, C-14), 37.71 (CH_3, C-20).

2-(3,6,9,12-Tetraoxapentadec-14-yn-1-yl)isoindoline-1,3-dione (24)

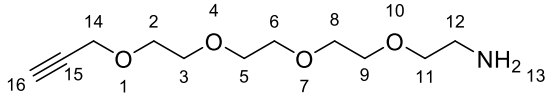


[361.39]

$C_{19}H_{23}NO_6$

For the next step 2.0 g 3,6,9,12-tetraoxapentadec-14-yn-1-ylmethanesulfonate **21** (6.4 mmol, 1 eq) was solved in 100 ml DMF in a 250 ml flask. After adding 1.79 g potassium phthalimide (9.7 mmol, 1.5 eq) the reaction was heated to 110°C. After 24 h the orange reaction solution was controlled by TLC (5% in DCM, UV and $KMnO_4$). The DMF was removed by an oil membrane pump. The brown solid was then re-dissolved with Et_2O and purified with column chromatography. The pure product were 1.8 g (79%) of a yellow oil. ^{1}H NMR (300 MHz, DMSO) δ ppm: 7.82 (s, 4H, H-21, H-22, H-23, H-24), 4.14 (d, *J* = 1.2 Hz, 2H, H-14), 3.69 (d, *J* = 4.8 Hz, 2H, H-12), 3.52 (d, *J* = 9.4 Hz, 12H, H-2, H-3, H-5, H-6, H-8, H-9), 3.41 (s, 1H, H-16). ^{13}C NMR (76 MHz, CDCl3) δ ppm: 167.96 (C_q, C-17, C-20), 132.82 (CH_{ar}, C-22, C-23), 131.88 (CH_{ar}, C-18, C-19), 74.41 (C_q, C-15), 70.30 (CH_2, C-5, C-6, C-8), 70.12 (CH_2, C-9), 69.85 (CH_2, C-3), 68.84 (CH_2, C-2), 67.63 (CH_2, C-11), 58.10 (CH_2, C-14), 37.04 (CH_2, C-12).

3,6,9,12-Tetraoxapentadec-14-yn-1-amine (25)



[231.29]

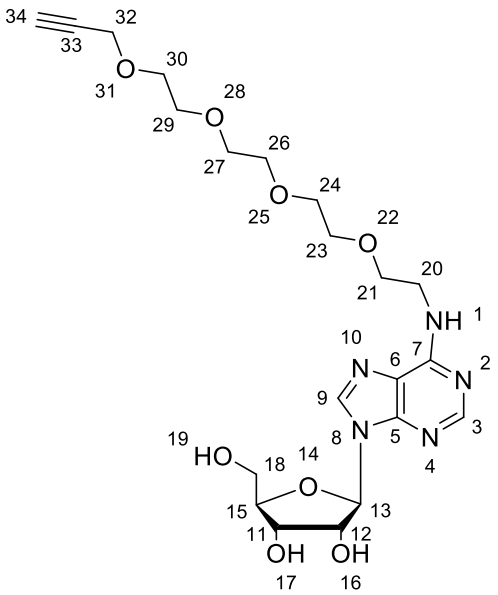
$C_{11}H_{21}NO_4$

Solving 1.0 g 2-(3,6,9,12-tetraoxapentadec-14-yn-1-yl)isoindoline-1,3-dione **22** (2.7 mmol, 1 eq) in 100 ml EtOH in a 250 ml two-neck round-bottom flask

and 2.9 ml hydrazine (3.0 g, 47 mmol, 17 eq) was added. The reaction mixture was stirred at 60°C. After 3 h a TLC control (5% MeOH in DCM) was done of the clear solution with white slime. The reaction mixture was concentrated to dryness. Purification of the product was done with column chromatography. 166 mg (26%) light brown oil resulted. ^{1}H NMR (300 MHz, DMSO) δ ppm: 4.14 (d, *J* = 2.2 Hz, 2H, H-14), 3.60 (t, *J* = 5.3 Hz, 3H, H-11), 3.56 – 3.51 (m, 12H, H-2, H-3, H-5, H-6, H-8, H-9), 3.33 (t, *J* = 6.6 Hz, 1H, H-16), 2.91 (t, *J* = 5.1 Hz, 2H, H-12).

HPLC-MS [*"CouBz_NO_Felix_095ML_MIN"*]: t_R = 3.180 min, [MS^+] = 232.

(2*R*,3*R*,4*S*,5*R*)-2-(6-((3,6,9,12-Tetraoxapentadec-14-yn-1-yl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (31)



[481.51]

$C_{21}H_{31}N_5O_8$

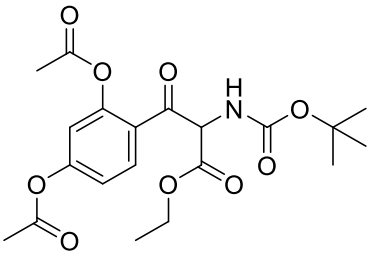
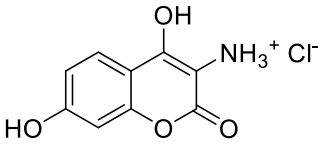
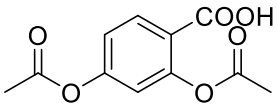
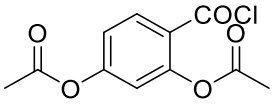
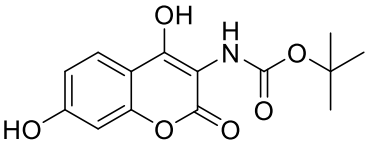
Putting 61.9 mg 6-chloropurine riboside **28** (216 µmol, 1 eq) in a 10-ml round-bottom flask. Adding 50 mg 3,6,9,12-tetraoxapentadec-14-yn-1-amine **23** (216 µmol, 1 eq) solved in 5 ml EtOH and 120.7 µl Et_3N (864.7 µmol, 4 eq). After adding 2 ml DMF and heating to 70°C everything was solved. Stirring the yellow solution at 70°C for 2 h. Removing the solvent under reduced pressure

and drying under vacuum. Purifying the crude product with column chromatography (15% MeOH in DCM) to achieve 400 mg (56%) white solid.

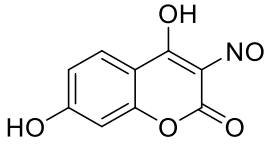
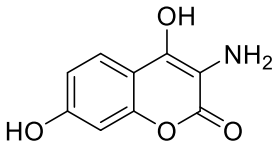
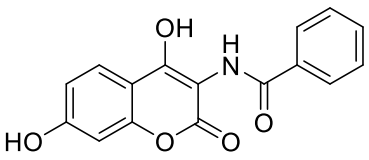
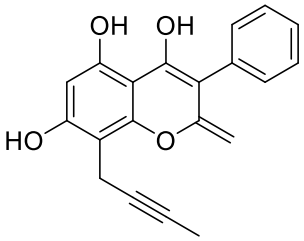
HPLC-MS [*"C8PEG_Bromoacetophe_095ml_min"*]: t_R = 2.513 min [MH^+] = 482.

8. List of Substrates

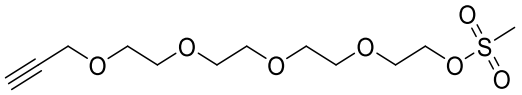
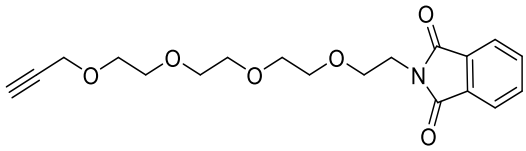
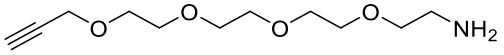
Synthesis II of CouBz

No.	Substrate	Structure	Amount
6 (KPL58)	4-(2-((*tert*-Butoxycarbonyl)amino)-3-ethoxy-3-oxopropanoyl)-1,3-phenylene diacetate		50 mg (in DMSO)
8	4,7-Dihydroxy-2-oxo-2*H*-chromen-3-aminium chloride		5.4 mg
8 (KPL60)			10.6 mg (in DMSO)
2 (KPL94)	2,4-Diacetoxybenzoic acid		1.0 g
3 (KPL81)	4-(Chlorocarbonyl)-1,3-phenylene diacetate		15 mg (in DMSO)
7 (KPL93)	*tert*-Butyl (4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)carbamate		35 mg (in DMSO)

Synthesis II of CouBz

No.	Substrate	Structure	Amount
14 (KPL112_NO)	4,7-Dihydroxy-3-nitroso-2*H*-chromen-2-one		40 mg (in DMSO)
14 (KPL116_NO)			15 mg (in DMSO)
15 (KPL112_NH_2)	3-Amino-4,7-dihydroxy-2*H*-chromen-2-one		30 mg (in DMSO) + 108 mg (dry)
15 (KPL116_NH_2)			23 mg (in DMSO)
9 (KPL115)	*N*-(4,7-Dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide		45 mg (in DMSO)
9 (KPL115)			100 mg (in DMSO)
9			53 mg (in DMSO)
KPL42	8-(but-2-ynyl)-4,5,7-trihydroxy-3-phenylcoumarin		1 mg

Immobilization of SAM

No.	Substrate	Structure	Amount
23 (KPL106)	3,6,9,12-Tetraoxapentadec-14-yn-1-ylmethanesulfonate		357 mg (in DMSO)
24 (KPL50)	2-(3,6,9,12-Tetraoxapentadec-14-yn-1-yl)isoindoline-1,3-dione		20 mg (in DMSO)
24 (KPL79)			36 mg (in DMSO)
25 (KPL83)	3,6,9,12-Tetraoxapentadec-14-yn-1-amine		50 mg (in 5 ml EtOH)
			42 mg (in DMSO)
			27 mg (in DMSO)
25 (KPL118)			28 mg (in DMSO)

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10. Abbreviations

Analytical methods

^{13}C-NMR	carbon NMR
^{1}H-NMR	proton NMR
APT	attached proton test
bs	broad singlet
d	doublet
dd	doublet of doublet
ec	endcapped
ESI	electron spray ionization
GC	gas chromatography
Hz	Hertz
J	coupling constant
m	multiplet
m/z	mass/charge ratio
MHz	Mega-Hertz
min	minute
MS	mass spectrometry
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
ppm	parts per million
q	quadruplet
R_f	ratio of fronts
rt	room temperature
s	singlet
t	triplet
TLC	thin layer chromatography
t_R	retention time
UV	ultraviolet
δ	chemical shift

Chemicals

Acetyl-CoA	Acetyl-Coenzyme A
AgOTf	silver trifuloromethanesulfonate
AcCN	acetonitrile
Ac_2O	acetic anhydride
ATP	adenosine triphosphate
Boc	*tert*-butoxycarbonyl
BSA	bovine serum albumin
BzCl	benzoyl chloride
CAM	cerium ammonium molybdate
$CDCl_3$	deuterated chloroform
CH	cyclohexane
Cou_2OH	2,4-dihydroxy-2*H*-chromen-2-one
CouBz	*N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide
DBU	1,8-diazabicycloundec-7-ene
DCM	dichloromethane
DIBAL-H	diisobutylaluminium hydride
DMAP	4-(dimethylamino)-pyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMSO-d_6	deuterated dimethylsulfoxide
DNA	2´-deoxyribonucleic acids
EE	ethyl acetate
Et_3N	triethylamine
Et_2O	diethylether
EtOH	ethanol
E.coli	Escherichia coli
FAD	flavin adenine dinucleotide
HCl	hydrochloride
HCOOH	formic acid
HNO_3	nitric acid
H_2SO_4	sulfuric acid

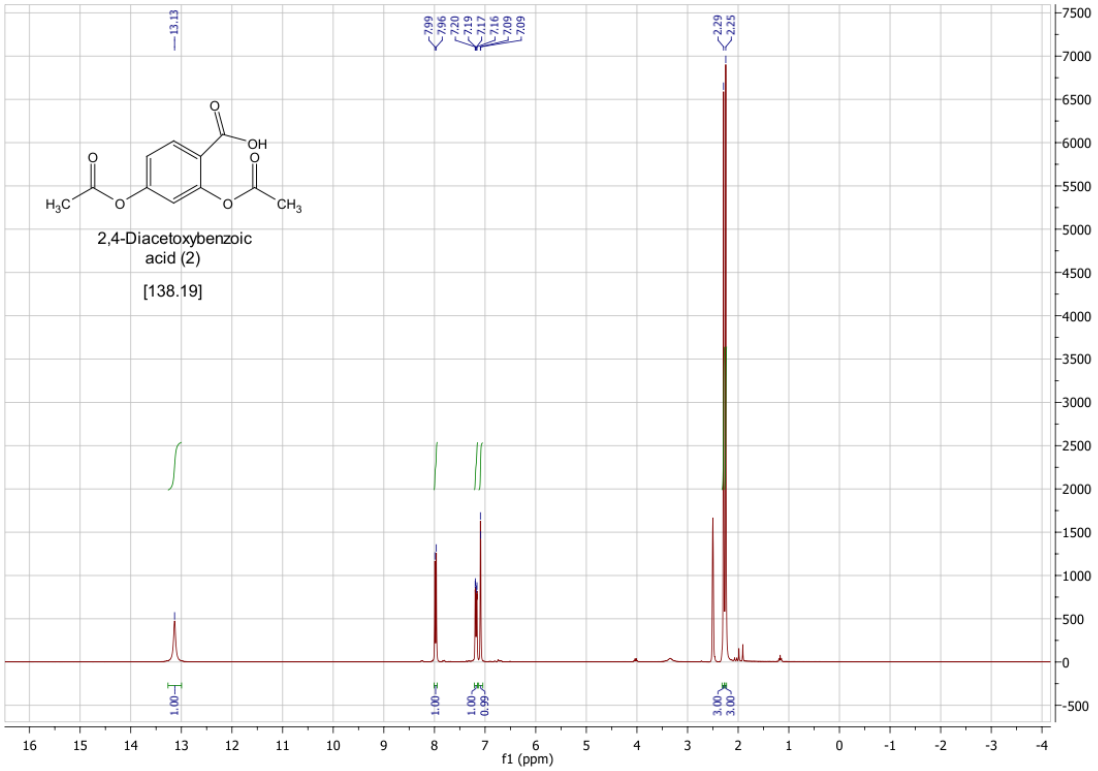
K_2CO_3	potassium carbonate
$KHSO_4$	potassium hydrogen sulfate
KI	potassium iodide
$KMnO_4$	potassium permanganate
KOH	potassium hydroxid
MeOH	methanol
$MgCl_2$	magnesium chloride
MsCl	methanesulfonyl chloride
Mtase	methyltransferase
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NaH	sodium hydride
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
Na_2SO_4	sodium sulfate
NH_4Cl	ammonium chloride
OEG	oligo(ethylene glycol)
PEG	poly(ethylene glycol)
Py	pyridine
RNA	ribonucleic acids
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-adenosyl-L-methionine
sat.	saturated
$SOCl_2$	thionyl chloride
*t*BuOH	*tert*-butyl alcohol
*t*BuONO	*tert*-butyl nitrite
THF	tetrahydrofuran
WT	wild type

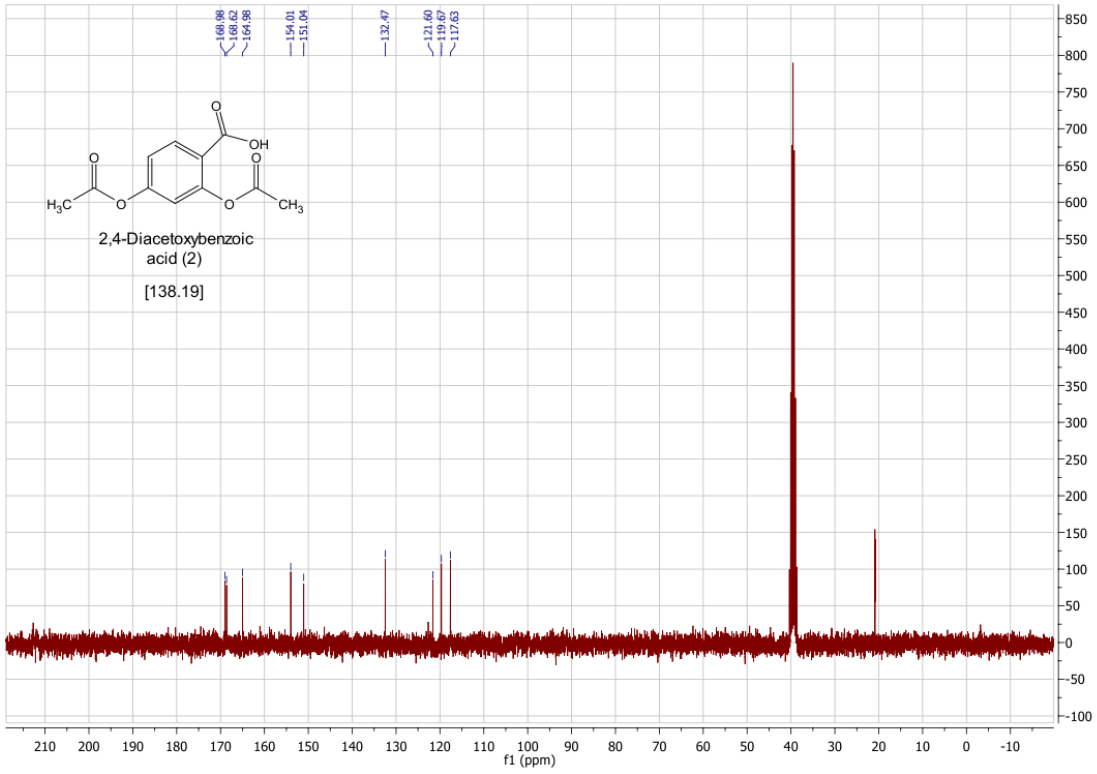
Others

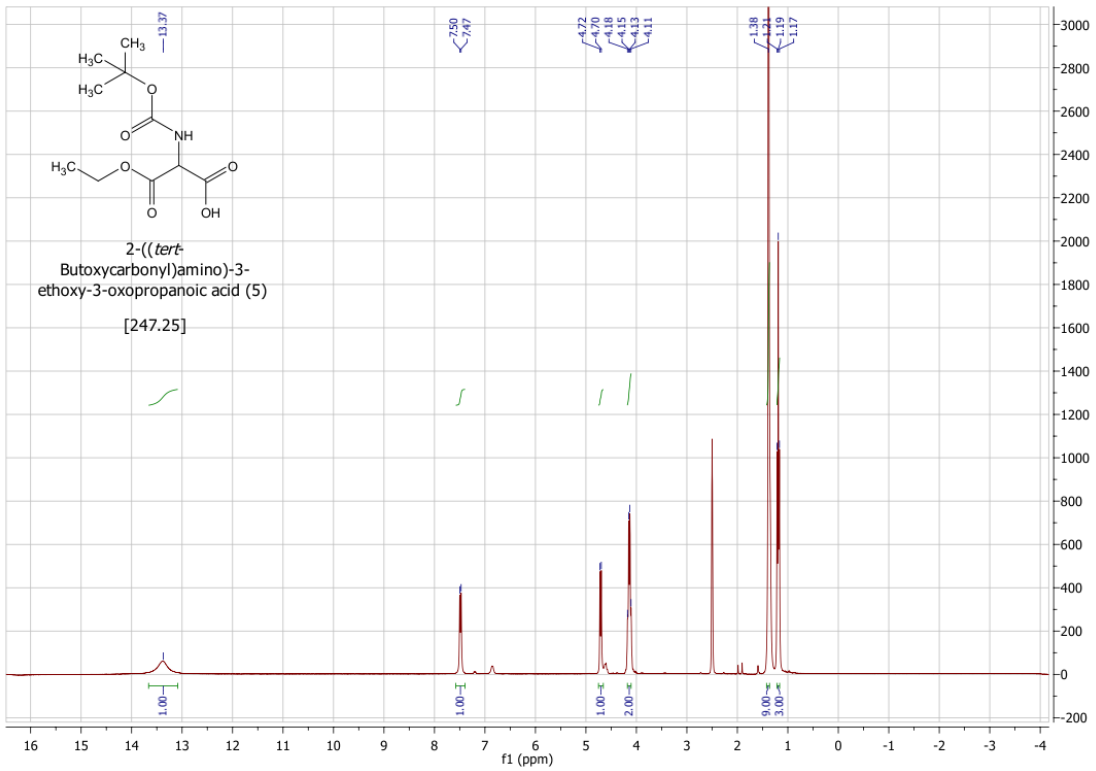
Å	angstrom
aq.	aqueous
%	percentage
°C	degree Celsius

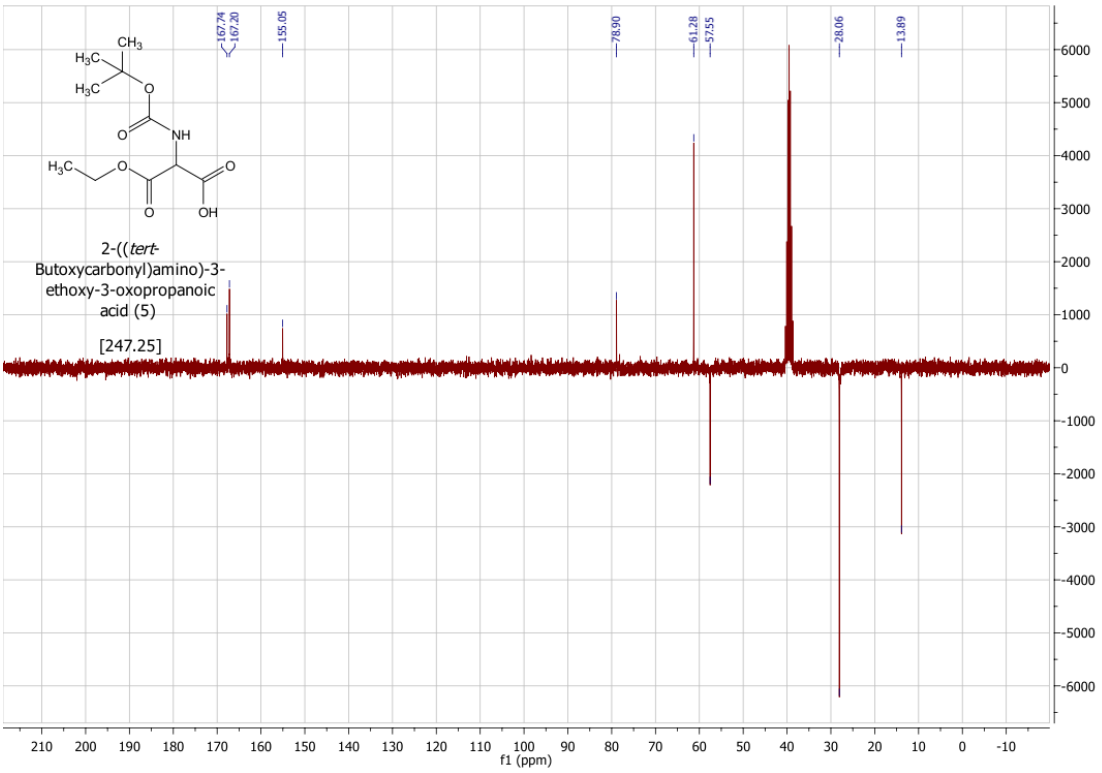
Da	Dalton
µg	microgramm
µL	microliter
µmol	micromol
µM	micromolar
cat.	catalytic
cm	centimeter
conc.	concentration
deion.	deionized
EC	enzyme class
eq	equivalent
et al.	et alii
Fig.	figure
g	gramm
kDa	kilodalton
M	molar
mg	milligramm
mL	milliliter
mmol	millimol
N	normal
nm	nanometer
pK_a	negative logarithmic acid dissociation
quant.	quantitative
rt	room temperature
S_EAr	electrophilic aromatic substitution
S_N2	bimolecular nucleophilic substitution
tert.	tertiary
(wt/wt)	weight/weight

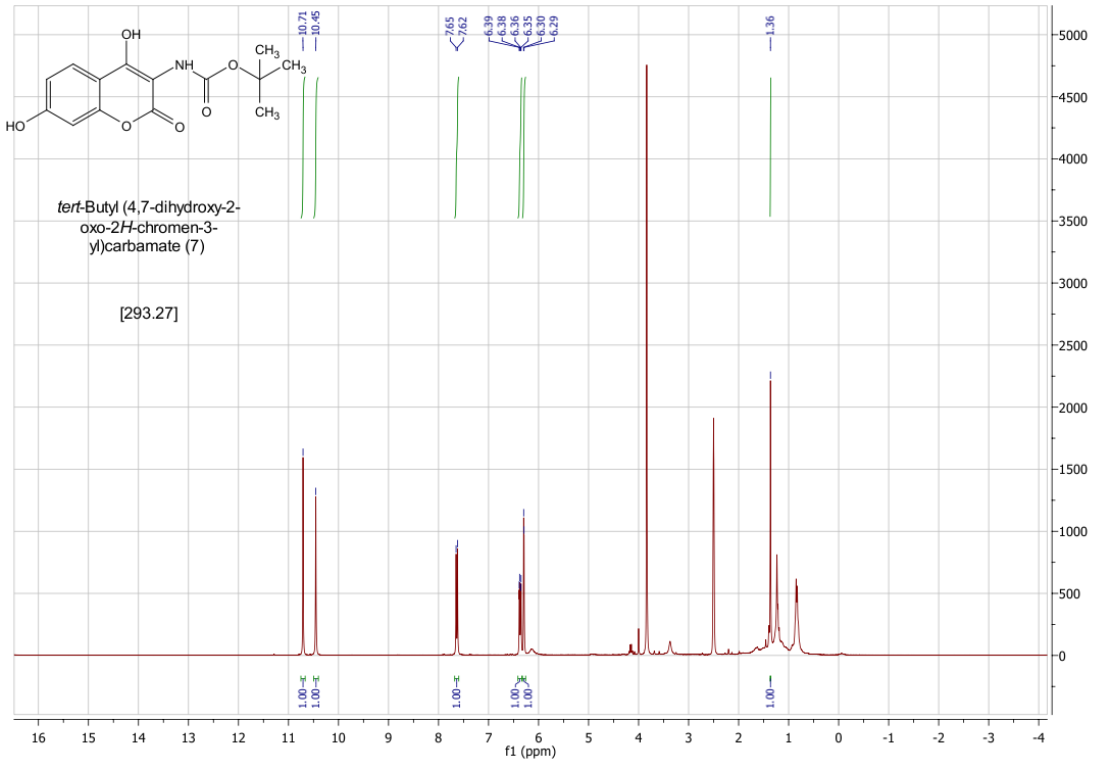
11. Appendix

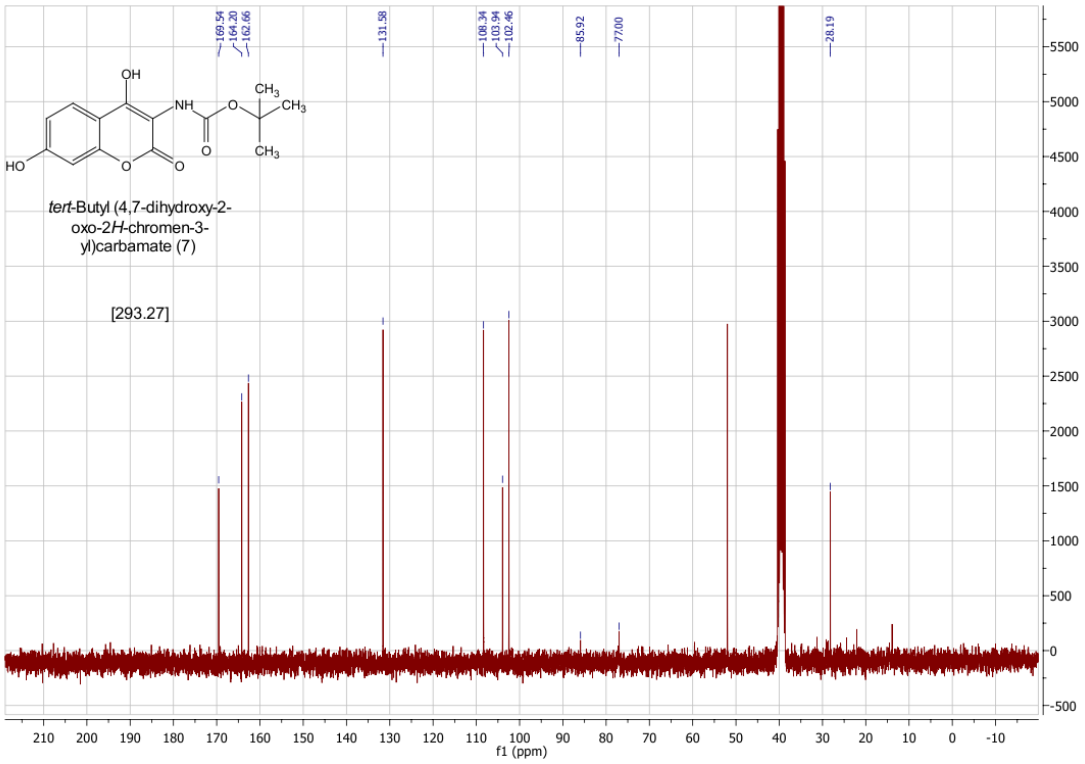

13.13
7.99
7.96
7.20
7.19
7.17
7.16
7.09
7.09
2.29
2.25
OH
O
O
O
O
H_3C
CH_3
2,4-Diacetoxybenzoic
acid (2)
[138.19]
1.00
1.00
1.00
0.99
3.00
3.00
f1 (ppm)

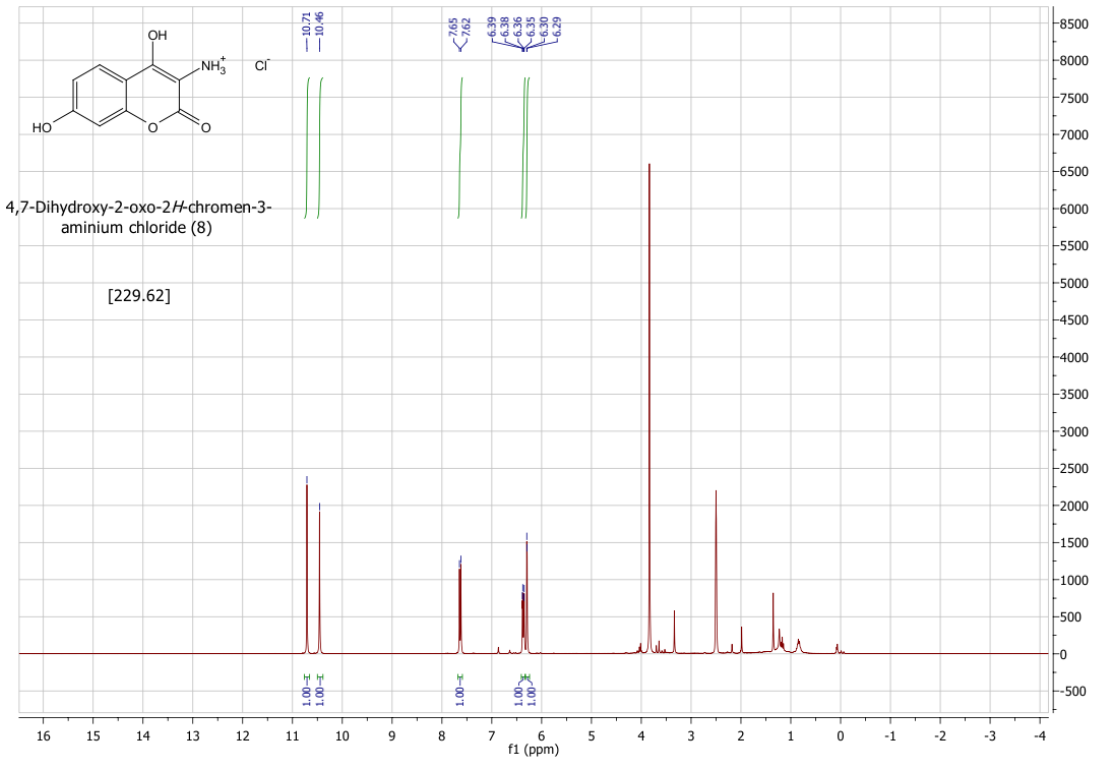

168.98
168.62
164.98
154.01
151.04
132.47
121.60
119.67
117.63
OH
O
O
O
O
H_3C
CH_3
2,4-Diacetoxybenzoic
acid (2)
[138.19]
f1 (ppm)

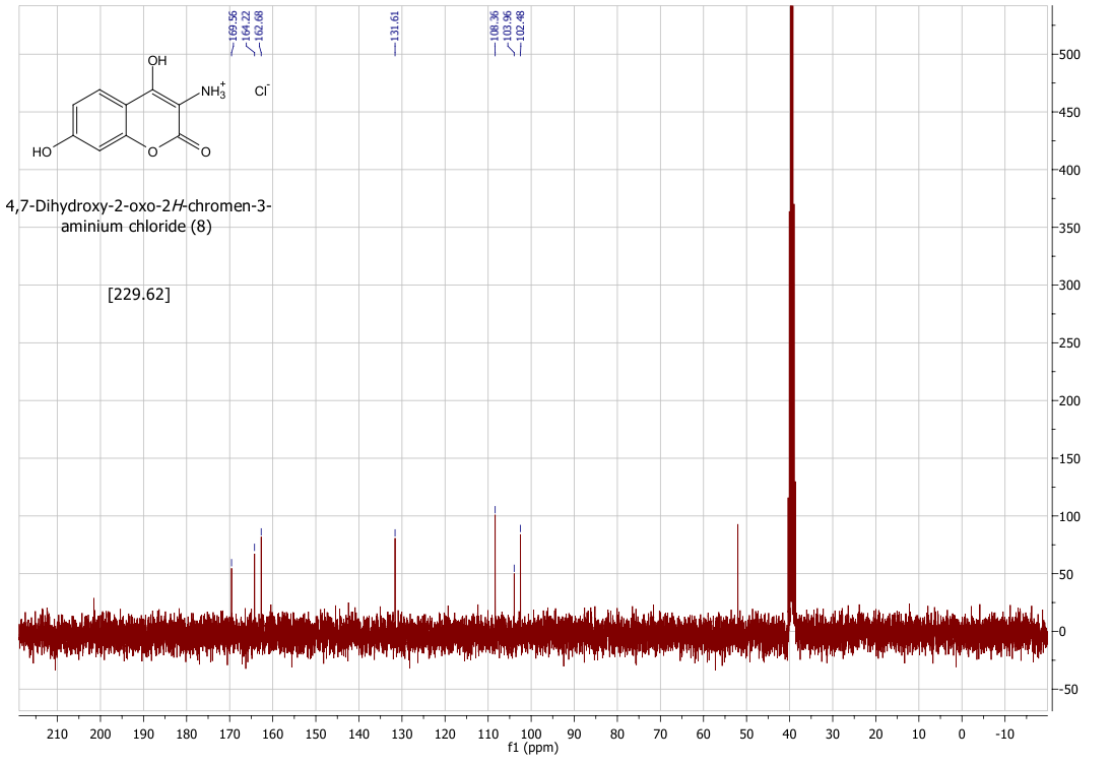

2-((tert-Butoxycarbonyl)amino)-3-ethoxy-3-oxopropanoic acid (5)
[247.25]
f1 (ppm)

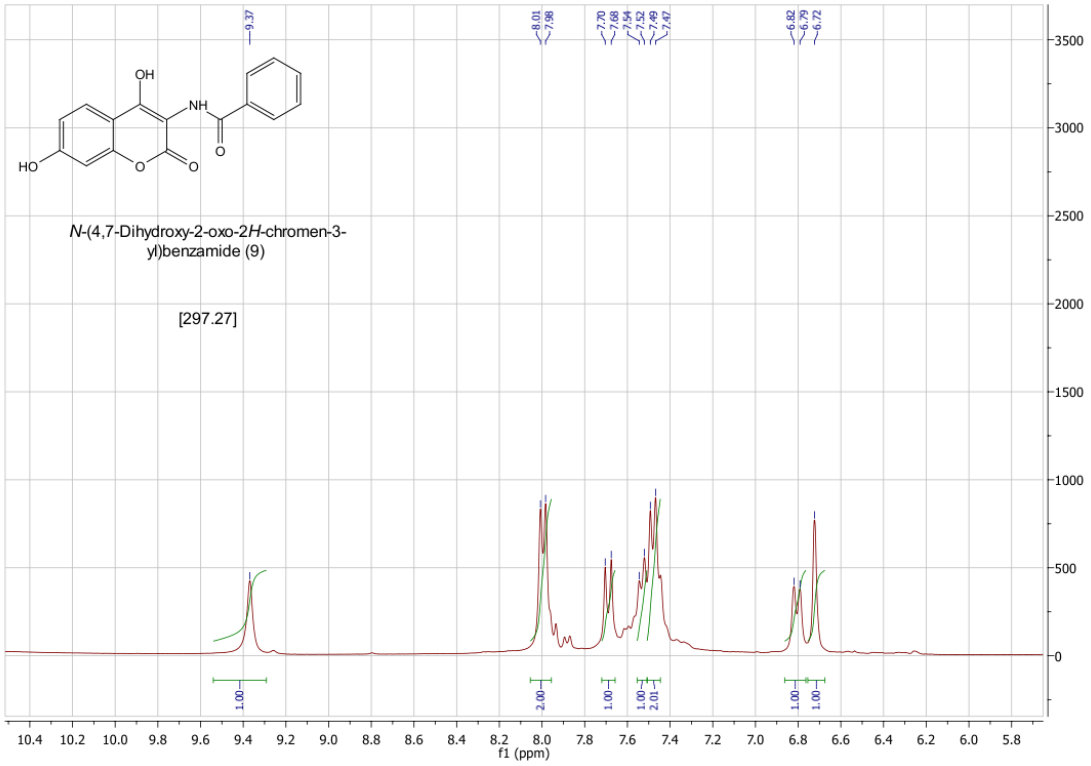

2-((tert-Butoxycarbonyl)amino)-3-ethoxy-3-oxopropanoic acid (5)
[247.25]
f1 (ppm)

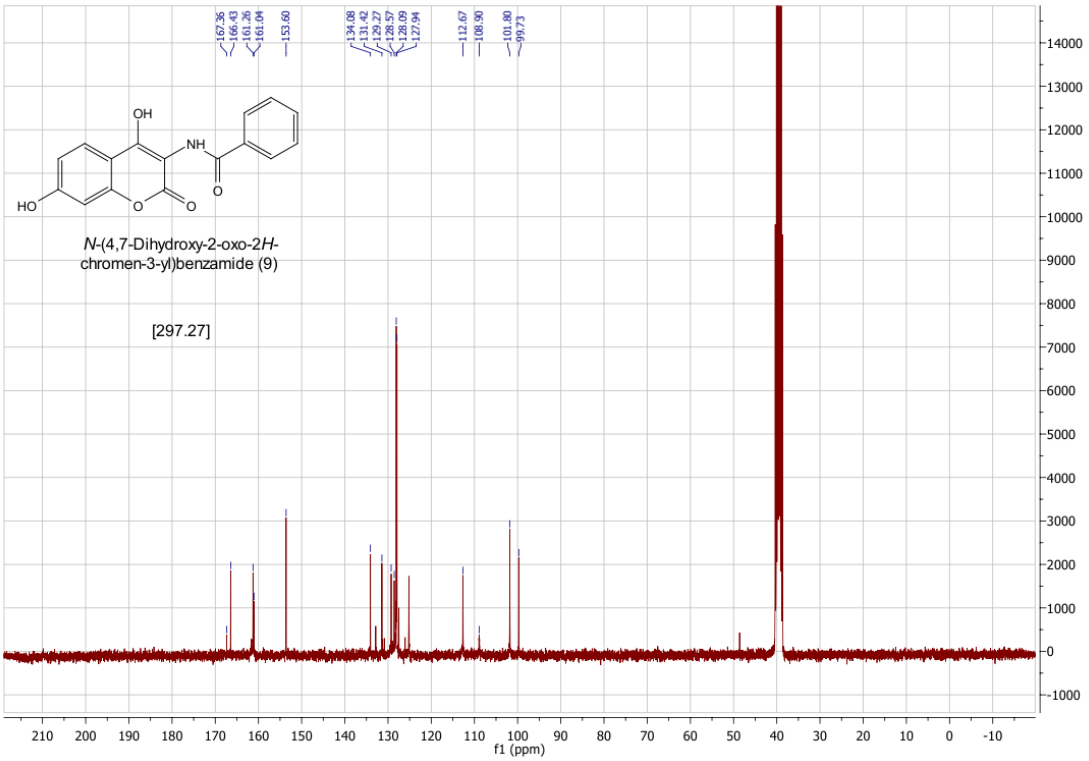

OH
NH
O
CH3
CH3
CH3
HO
O
O
O
tert-Butyl (4,7-dihydroxy-2-oxo-2H-chromen-3-yl)carbamate (7)
[293.27]
10.71
10.45
7.65
7.62
6.39
6.38
6.36
6.35
6.30
6.29
1.36
1.00
f1 (ppm)

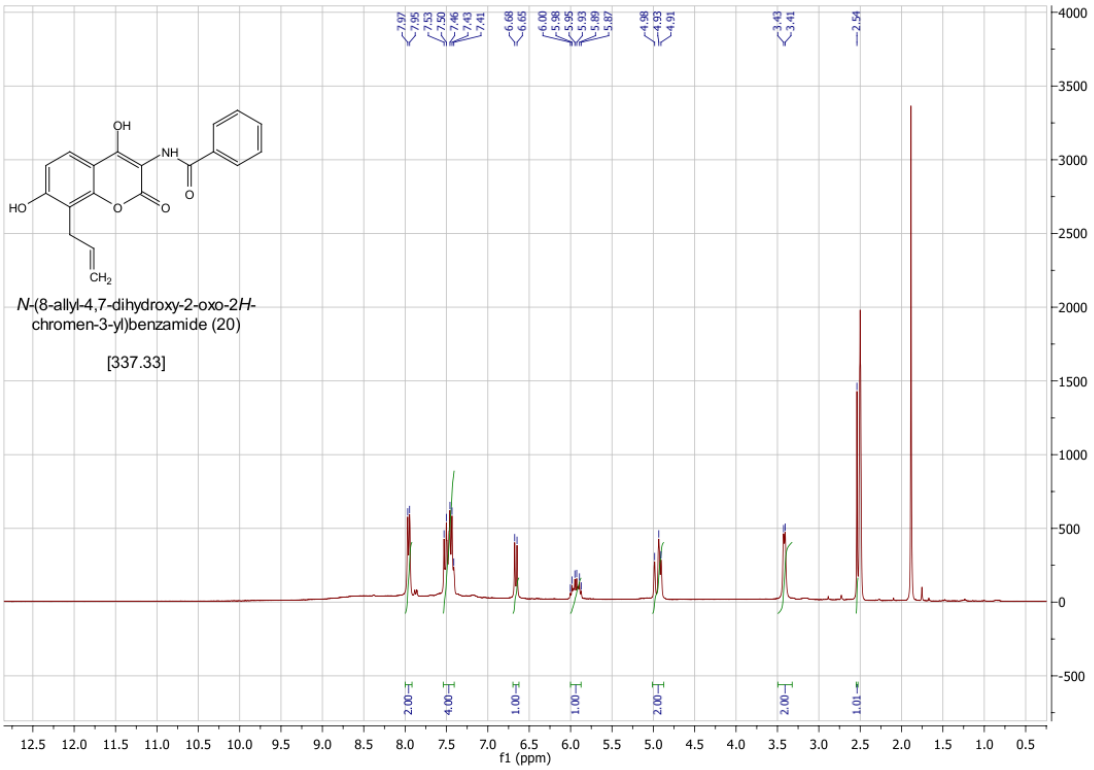

OH
NH
O
CH3
CH3
CH3
HO
O
O
O
tert-Butyl (4,7-dihydroxy-2-oxo-2H-chromen-3-yl)carbamate (7)
[293.27]
169.54
164.20
162.66
131.58
108.34
103.94
102.46
85.92
77.00
28.19
f1 (ppm)

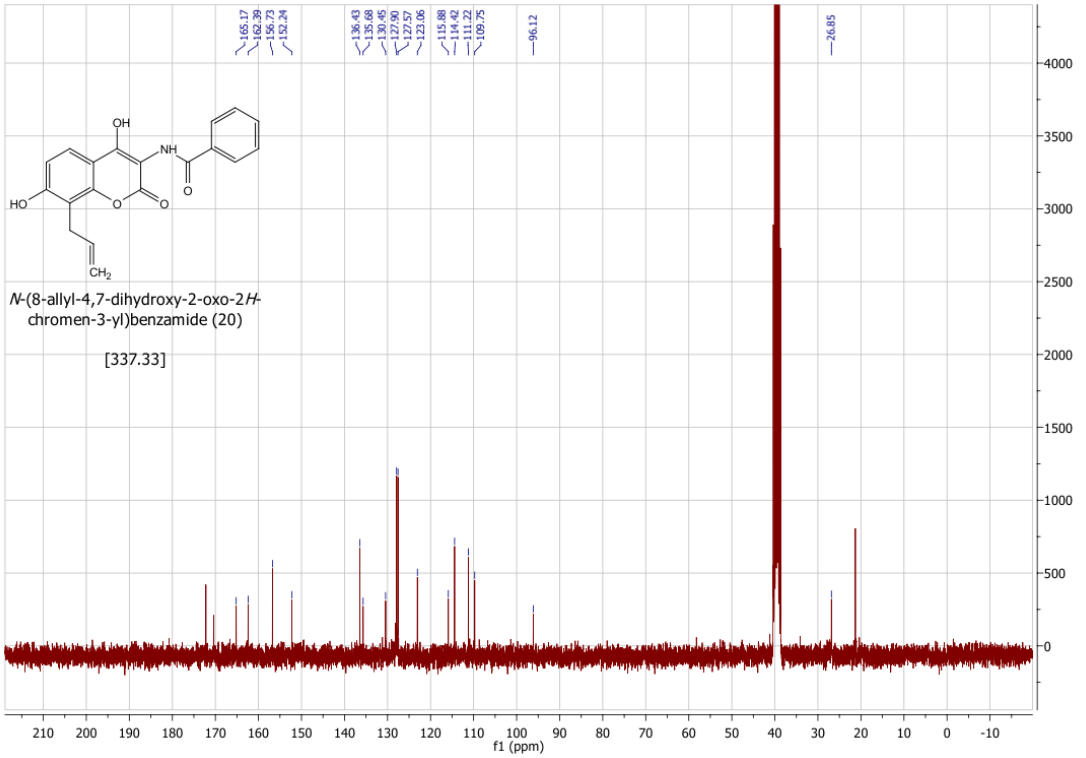

OH
NH3+
Cl-
HO
O
O
4,7-Dihydroxy-2-oxo-2H-chromen-3-aminium chloride (8)
[229.62]
10.71
10.46
7.65
7.62
6.39
6.38
6.36
6.35
6.30
6.29
1.00
1.00
1.00
1.00
1.00
f1 (ppm)

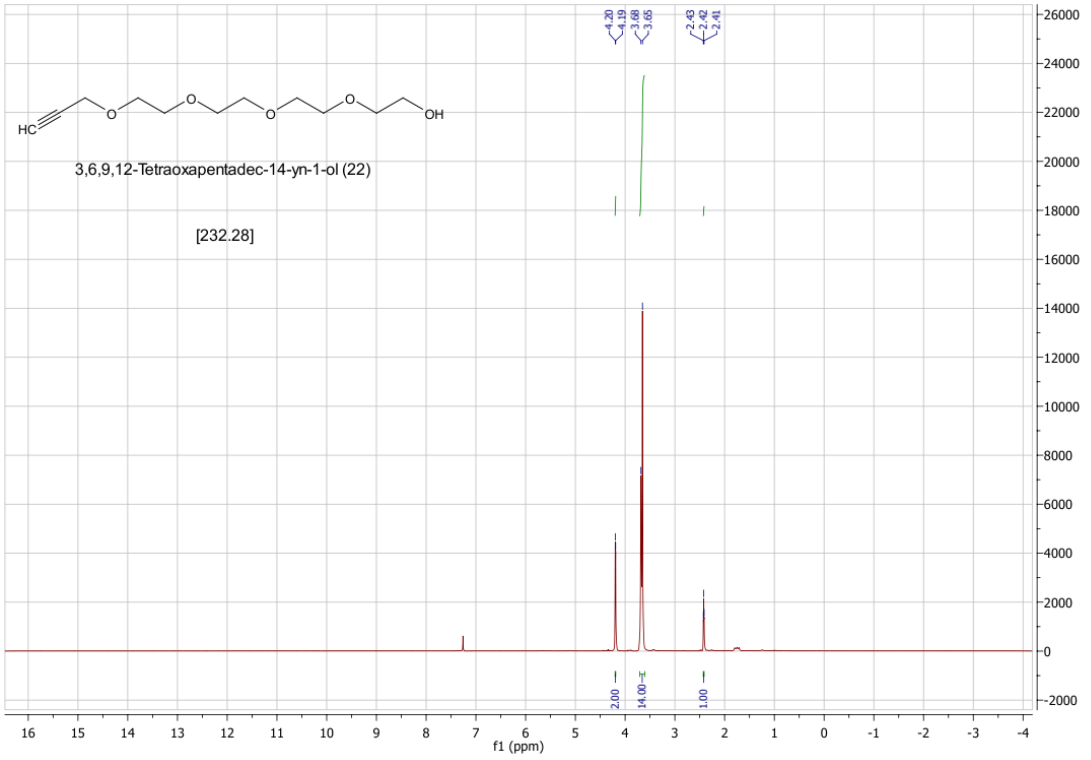

OH
NH3+
Cl-
HO
O
O
4,7-Dihydroxy-2-oxo-2H-chromen-3-aminium chloride (8)
[229.62]
169.56
164.22
162.68
131.61
108.36
103.96
102.48
f1 (ppm)

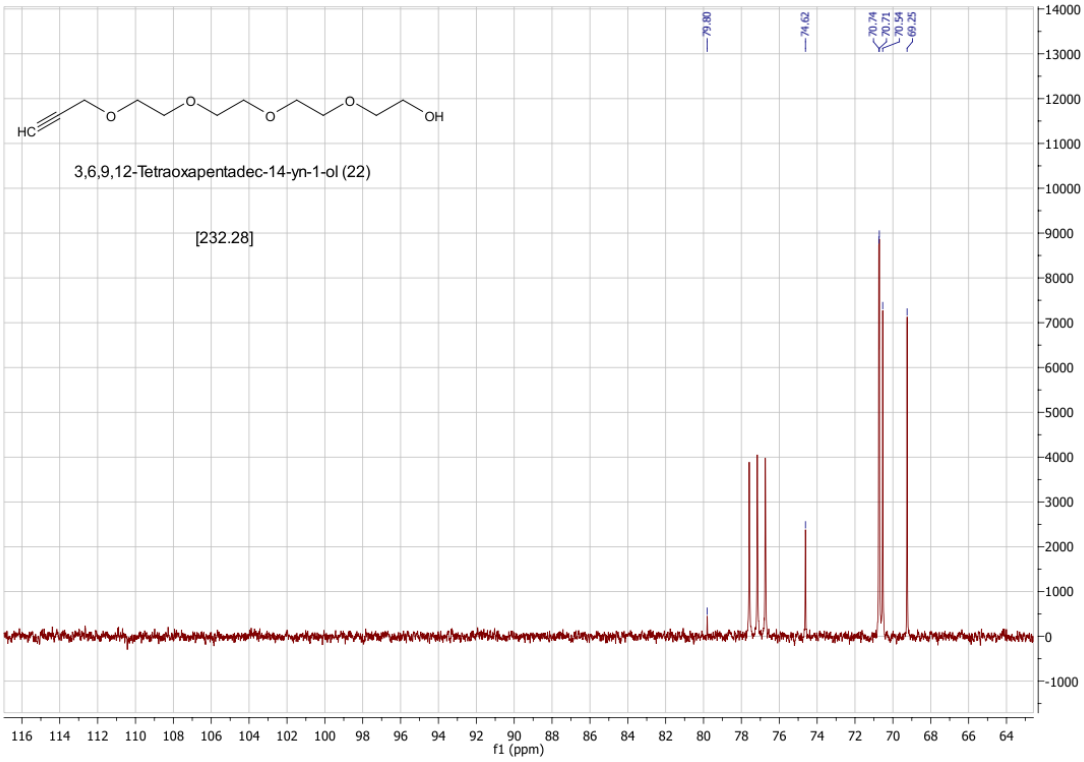

OH
NH
O
HO
O
O
N-(4,7-Dihydroxy-2-oxo-2H-chromen-3-yl)benzamide (9)
[297.27]
f1 (ppm)

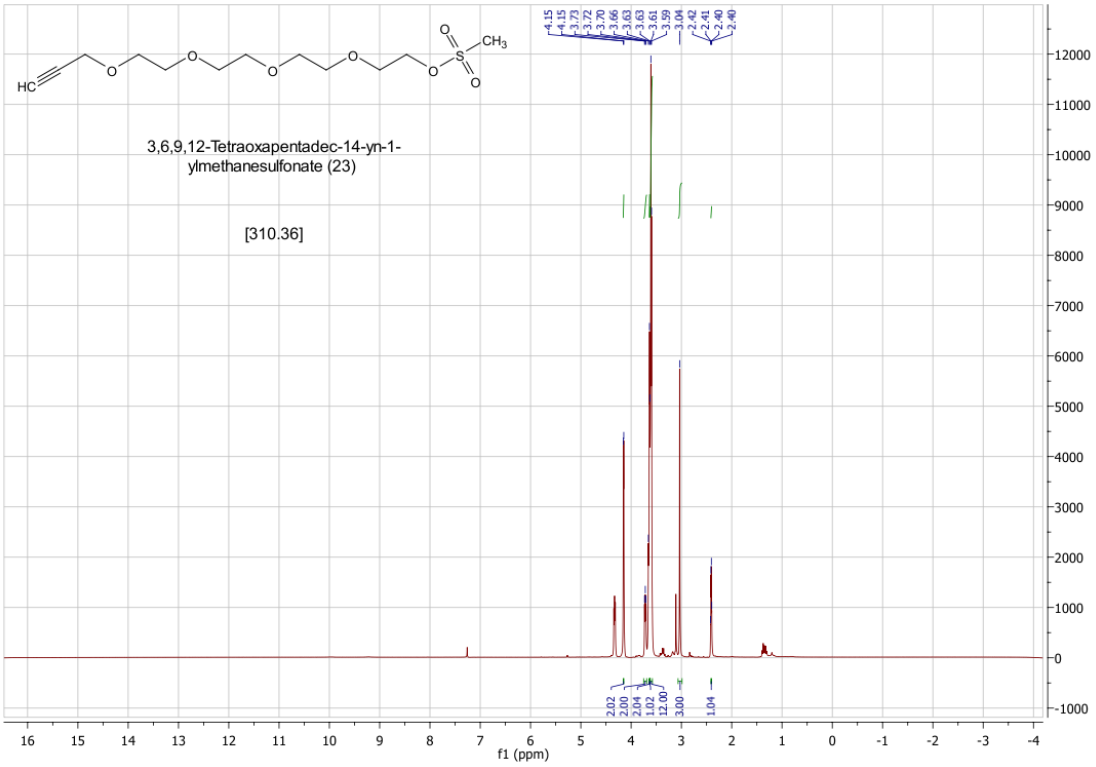

OH
NH
O
HO
O
O
N-(4,7-Dihydroxy-2-oxo-2H-chromen-3-yl)benzamide (9)
[297.27]
f1 (ppm)

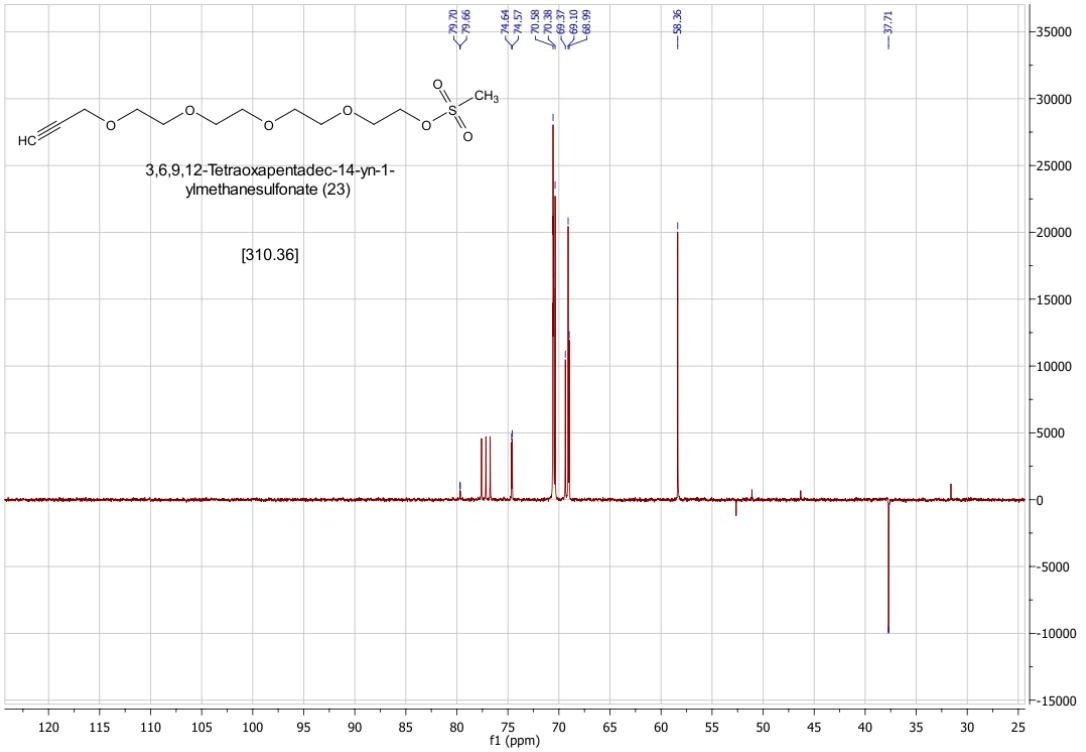

OH
NH
HO
O
O
O
CH2
N-(8-allyl-4,7-dihydroxy-2-oxo-2H-chromen-3-yl)benzamide (20)
[337.33]
f1 (ppm)

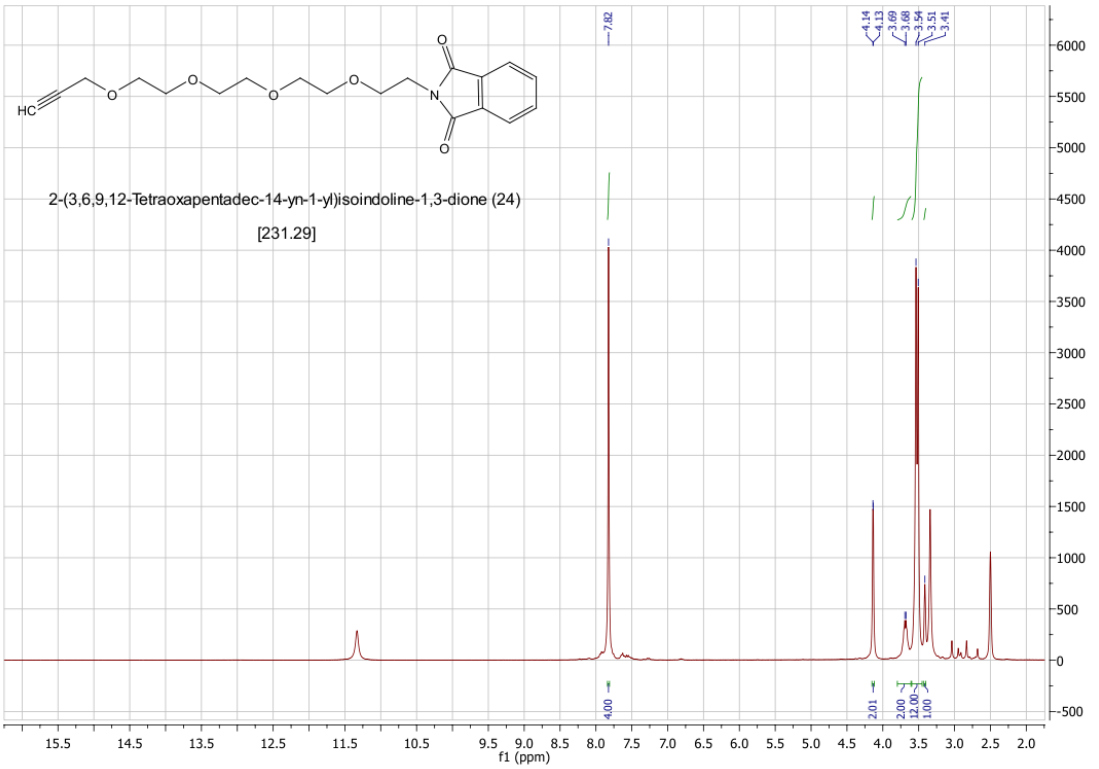

OH
NH
HO
O
O
O
CH2
N-(8-allyl-4,7-dihydroxy-2-oxo-2H-chromen-3-yl)benzamide (20)
[337.33]
f1 (ppm)

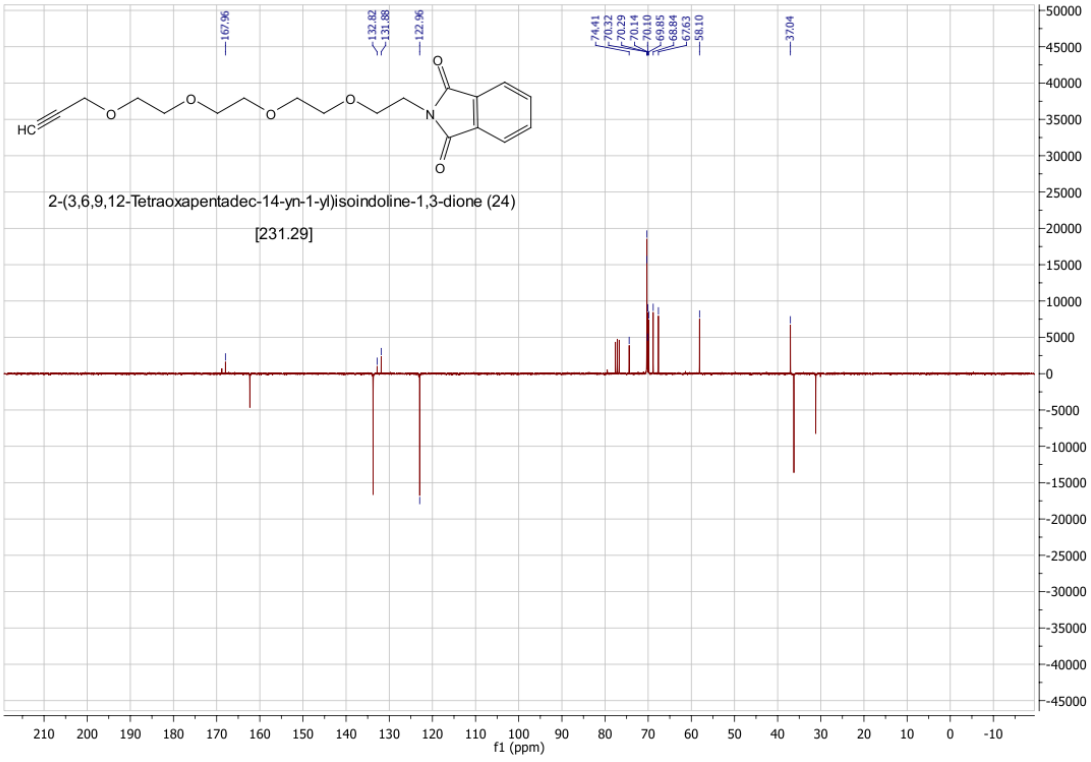

HC
O
O
O
O
OH
3,6,9,12-Tetraoxapentadec-14-yn-1-ol (22)
[232.28]
4.20
4.19
3.68
3.65
2.43
2.42
2.41
2.00
14.00
1.00
f1 (ppm)

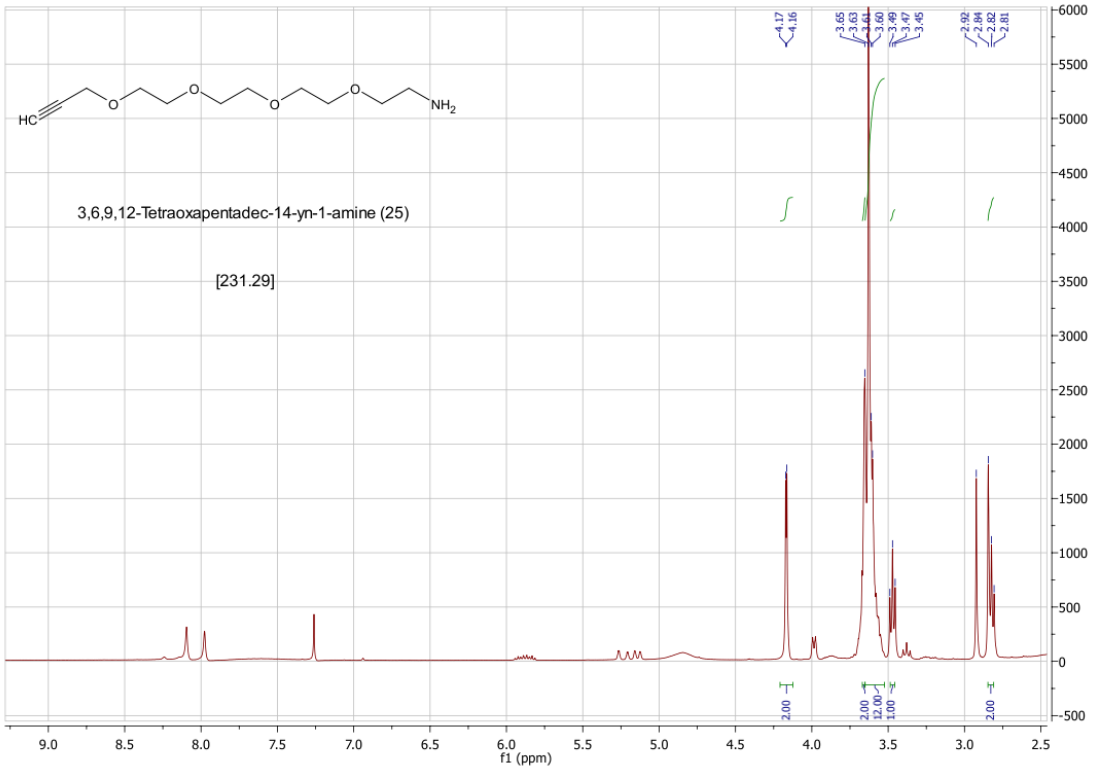

HC
O
O
O
O
OH
3,6,9,12-Tetraoxapentadec-14-yn-1-ol (22)
[232.28]
79.80
74.62
70.74
70.71
70.54
69.25
f1 (ppm)


3,6,9,12-Tetraoxapentadec-14-yn-1-ylmethanesulfonate (23)
[310.36]
f1 (ppm)


3,6,9,12-Tetraoxapentadec-14-yn-1-ylmethanesulfonate (23)
[310.36]
f1 (ppm)


2-(3,6,9,12-Tetraoxapentadec-14-yn-1-yl)isoindoline-1,3-dione (24)
[231.29]
f1 (ppm)


2-(3,6,9,12-Tetraoxapentadec-14-yn-1-yl)isoindoline-1,3-dione (24)
[231.29]
f1 (ppm)


HC
O
O
O
O
NH2
3,6,9,12-Tetraoxapentadec-14-yn-1-amine (25)
[231.29]
4.17
4.16
3.65
3.63
3.61
3.60
3.49
3.47
3.45
2.92
2.84
2.82
2.81
2.00
2.00
12.00
1.00
2.00
f1 (ppm)