New approaches for flavoenzyme applications

Krzek, Marzena

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CHAPTER 3

SYNTHESIS OF A NOVEL FLAVIN COFACTOR ANALogue, $N^6$-((BUTYL-2-EN-4-AMINE))-FAD FOR ENZYMES IMMOBILIZATION

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Abstract

Flavin-containing enzymes can be used in various biotechnological applications. For that, their immobilization is advantageous and often required for a cost-effective process. We have recently shown that flavoenzymes can be immobilized via their flavin adenine dinucleotide (FAD) cofactor. This approach utilizes FAD derivatized at the adenine part with a linker containing a specific functional group, which can form covalent bond with a target material. The FAD-decorated material can serve for cofactor-mediated immobilization of apo flavoenzymes yielding tightly immobilized, stable and active biocatalysts (Krzek et al., 2016). A limitation for this approach is the accessibility of a suitable FAD analogue. Currently known synthesis procedures are laborious, time consuming and inefficient.

In this chapter, a new method for the efficient preparation of a novel FAD analogue substituted at the adenine moiety (N<sup>6</sup>-(butyl-2-en-4-amine)-FAD) is described. The FAD analogue contains an aliphatic linker with a terminal primary amine which makes it suitable for cofactor-mediated flavoenzyme immobilization. The FAD analogue was prepared, starting from FAD, with 40% yield. The final structure of the FAD analogue was confirmed using spectroscopic and spectrometric methods (NMR, UV-Vis, MS) and cyclic voltammetry. Furthermore, the compound was successfully applied for FAD-mediated immobilization of the flavoenzyme phenylacetone monooxygenase.

Key words: FAD, immobilization, Dimroth rearrangement, NMR, alkylation
1. INTRODUCTION

Flavin adenine dinucleotide (FAD)-containing enzymes are regarded as particularly useful as oxidative biocatalysts in the pharmaceutical, food and fine-chemical industry (van Berkel et al. 2006) and they can be also exploited for biosensors (Posthuma-Trumpie et al., 2007). To enable such biotechnological applications, an efficient and robust immobilization of a flavoenzyme is often essential. We have recently reported on a new method for the FAD-mediated flavoenzyme attachment to a solid carrier (Krzek et al., 2016). This approach exploits the common characteristics of flavoenzymes that (1) the adenosine part of FAD is close to the protein core surface, while (2) the affinity of this cofactor towards apo flavoenzymes is very high. By using a FAD derivative that has been modified at the adenine moiety, e.g. having a linker with a terminal functional group, covalent attachment of the flavin cofactor to a carrier can be achieved. Subsequently, such a FAD-decorated material can serve for reconstitution of apo flavoenzymes yielding fully active, robustly immobilized holo flavoenzymes (Krzek et al., 2016). Importantly, through the use of different linkers, this approach allows tuning the distance and orientation between the enzyme and surface. The key compound in this approach is an FAD analogue modified at the adenosine moiety (Stocker et al. 1996; Krzek et al. 2016; Hefti et al. 2003; Hefti, Vervoort and Van Berkel, 2003; Hefti, Vervoort and Van Berkel, 2003). However, such FAD derivatives are not commercially available and their synthesis using previously described protocols is laborious and inefficient.

Several methods to prepare FAD derivatives for covalent coupling to a surface and subsequent enzymatic reconstitution were already described in the 80’s (Zappelli et al., 1978; Wingard, 1984). Originally, those cofactor analogues were studied with the FAD-containing glucose oxidase from Aspergillus niger. The low yields of reconstitution of this enzyme and difficulties in apo enzyme preparation inhibited further development of flavin-mediated immobilizations and, related to that, synthesis routes for obtaining relevant FAD analogues (Wingard, 1984). FAD modifications can change the functional properties of the cofactor, which would alter flavoenzyme characteristic as well. When derivatized at the isoalloxazine ring, it typically alters the redox potential and the spectral properties (Nishina et al., 2003; Roth et al., 2004). Additionally, this part of FAD is located within the protein core and may prevent apo protein reconstitution or trigger detrimental conformational changes in the protein (Wingard, 1984; Nishina et al., 2003; Roth et al., 2004). In contrast to that, substitution at the adenosine part does not influence the redox properties of the isoalloxazine moiety and is often compatible with apo protein reconstitution because the adenosine moiety is often very close to the protein surface (Stocker, Hecht and Bückmann, 1996). The adenosine part can be alkylated at positions 1 and 6. To obtain $N^6$-alkylated adenosine, nitrogen at position 6 may first undergo halogenation (Ikehara, Ogiso and Maruyama, 1977). However, such modification typically requires harsh conditions under which the FAD structure will not be preserved. As alternative approach, the 1 position may be targeted, as it is more reactive when compared with $N^6$. While $N^1$ analogues have
been reported as relatively unstable (Bückmann, Wray and Stocker, 1997), the analogues alkylated at position 1 can rearrange into $N^6$ derivatives via a so-called Dimroth rearrangement. Therefore, for obtaining generally applicable FAD analogues with preserved catalytic properties, the most preferable way is to obtain the $N^6$ analogues via Dimroth rearrangement.

Until now, not many studies on synthesis of $N^6$-FAD analogues have been reported. The first published strategy was to substitute the $N^6$ position of adenosine and subsequently build up the complete FAD molecule by coupling it to the flavin mononucleotide (FMN) (Morris and Buckler, 1983; Stocker, Hecht and Bückmann, 1996). Functionalized $N^6$ substituted adenosine compounds can be further modified in order to obtain the desired FAD analogue (Stocker, Hecht and Bückmann, 1996). Nevertheless, this synthesis pathway involves multiple steps starting from very costly FMN giving only 17-20% overall yield (Stocker, Hecht and Bückmann, 1996). Another, simpler approach involves only two steps and uses FAD as a starting compound (Scheme 1). First, the selective alkylation at the FAD adenosine 1 position is performed. This step is followed by a Dimroth rearrangement into a more stable, adenosine $N^6$ FAD derivative (van der Plas et al., 1995). The method has been patented in 1995 (Bückmann et al., 1995). Herein, we describe a more efficient approach to prepare a $N^6$-FAD analogue and compare it with the approach reported by Bückmann.

The largest bottleneck of the first step of the published synthesis route is the very low rate of alkylation at position 1 of adenosine. In the published method it takes around 3 days (Bückmann, Wray and Stocker, 1997) (Scheme 1). The second step, rearrangement from position 1 into $N^6$ in the method described by Bückmann suffers from the formation of significant amounts of side products as a result of reactions of the linker’s terminal, reactive group, which may lead *inter alia* to cyclisation into the 1,1$^6$-ethanoadenosine derivative (van der Plas, Henk C. Bückmann and Wray, 1995). Moreover, purification of the sample after both steps is laborious and harmful for the products. The overall yield after two steps reaches only 24% (Bückmann, Wray and Stocker, 1997). We attempted to improve both steps for the preparation of a $N^6$-FAD analogue which contains a short linker moiety and

**Scheme 1.** Synthesis of $N^6$-substituted FAD analogue as patented in 1995 by Bückmann. Atom numbering at the adenine moiety is indicated with blue numbers.
a primary amine: \(N^6\)-\((\text{butyl}-2\text{-en}-4\text{-amine})\)\)-FAD. The amine can be exploited for selective, covalent coupling of the FAD analogue to a carrier material or other purposes.

We have attempted to optimize the alkylation at the position 1 of adenosine based on the recently published work on adenosine in DMF as a solvent. It was shown that the addition of salts such as KI and BaCO\(_3\) during alkylation with alkyl halides increases the efficiency (Oslovskya, Drenicheva. and Mikhailova, 2015). Additionally, the use of allylic halides may be more powerful than the use of saturated alkyl halides, as the double bond increases its reactivity. Furthermore, iodides (that can be obtained via Finkelstein reaction) provide a very good leaving group (Finkelstein, 1910). Importantly, a similar approach has been recently explored for the synthesis of a natural product from \(M.\) \(\text{tuberculosis}\). Alkylation of adenosine was performed at room temperature overnight with NaI in DMF, which resulted in a yield of 76% (Buter \textit{et al.}, 2016). As FAD is a more fragile molecule and does not dissolve in organic solvents, the same conditions cannot be applied. It dissolves well in water but an aqueous environment will slow down the alkylation. Therefore, we used a mixture of solvents (DMSO:DMF:H\(_2\)O). For the alkylation reaction, together with the aforementioned salts, we employed an allyl bromide as it is a more reactive electrophile than several other allylic halides. Moreover, the reaction was performed at 50 °C, which in case of the patented approach was not possible due to the low boiling point of the employed alkylation agent. For simplicity of the product identification, preliminary tests were performed on adenosine with 4-\((\text{Fmoc-amino})\)-1-bromobut-2-ene as the alkylation agent.

The Dimroth rearrangement of adenosine alkylated at position 1 into position \(N^6\) is a nucleophile-assisted process, which involves ring opening and dissociation of a proton (van der Plas \textit{et al.}, 1995). The literature reports a high rearrangement yield for the prepared aminoethyl FAD derivative and low efficiency when using a carboxyl analogue (Bückmannn, 1995). It also suggests that the amine moiety can act as an inter- or less probably intramolecular nucleophile, and indicates a strong dependency of the Dimroth rearrangement on the nucleophile assistance. For this step, Bückmann \textit{et al} employed LiOH (Bückmann, Wray and Stocker, 1997), which is strong base, and the hydroxide anion is relatively strong nucleophile. FAD is unstable at extreme pH values due to the labile pyrophosphate moiety which prevents exploiting hydroxides as nucleophiles for the Dimroth rearrangement (Bückmannn, 1995). We employed a compound which is a weak base and strong nucleophile at the same time; diethylamine. Another aspect to be improved compared to described methods is to reduce side product formation, which are mostly related to the terminal primary amine reactivity. For this reason, we blocked the amine with a protecting group cleavable under the Dimroth rearrangement conditions.

The newly developed synthetic procedure has been applied for the preparation of a new FAD derivative: \(N^6\)-\((\text{butyl}-2\text{-en}-4\text{-amine})\)\)-FAD. Moreover, the utility of this compound for cofactor-mediated immobilization of a flavoenzyme was demonstrated.
2. RESULTS AND DISCUSSION

2.1 TESTING A MODEL REACTION – ALKYLATION OF ADENOSINE

FAD is an expensive and relatively complex molecule. Therefore, for optimization of reaction conditions we first tested alkylation of the adenosine position 1 of the model compound adenosine. The alkylation agent 4-(Fmoc-amino)-1-bromobut-2-ene has been synthesized according to Scheme 2 and used for adenosine alkylation (Scheme 3). The preparation details and data are given in the experimental section. The approach taken suffered from a low overall yield (7%). The major limitation was the difficult purification after the second step as the final product was obtained as a dense oil and was hard to purify. Due to that, the planned pathway could still be performed from the very cheap reagents, nonetheless very large scale was needed to obtain efficient amount for alkylation of FAD on a gram scale.

Scheme 2. Synthesis of the alkylation agent for adenosine derivatization (4)

Scheme 3. Alkylation of adenosine at the position 1 with 4-(Fmoc-amino)-1-bromobut-2-ene was a model reaction for FAD alkylation.

SYNTHESIS OF N1-4-(Fmoc-Amino)-1-BROMOBUT-2-ENE ADENOSINE

The alkylation agent 4 was reacted with adenosine (Scheme 3) in DMF or in the SolventMix (DMSO:DMF:H2O (2:1:1 v/v/v)). The effect of time and addition of salts (BaCO3, NaI) on the yield was investigated as presented in Table 21. All reactions were performed at room temperature or 50 °C using 50 W microwave irradiation. Moreover, the addition of a huge excess of the non-nucleophilic
base 1,8-diazabicycloundec-7-ene (DBU) was tested. The results of the optimization are shown in Table 1 and an example of a \(^1\)H NMR spectrum is shown in Figure 1.

**Table 1. Conditions tested for adenosine alkylation optimization. Yields were calculated from \(^1\)H NMR spectra based on integrations of signals from the sugar anomeric centre.**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Solvent</th>
<th>Time</th>
<th>Molar equivalents of NaI + BaCO(_3)</th>
<th>Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>room temperature</td>
<td>DMF</td>
<td>20 h</td>
<td>1.2</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 days</td>
<td>1.2</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>SolventMix</td>
<td>2 days</td>
<td>1.2</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 days</td>
<td>2.4</td>
<td>47</td>
</tr>
<tr>
<td>50°C microwave</td>
<td>DMF</td>
<td>1 h</td>
<td>1.2</td>
<td>36</td>
</tr>
<tr>
<td>50W</td>
<td></td>
<td>3 h</td>
<td>1.2</td>
<td>45</td>
</tr>
<tr>
<td>4 days (RT) +1 h microwave</td>
<td>SolventMix</td>
<td>2.4 + 10 DBU</td>
<td>unspecific products</td>
<td></td>
</tr>
</tbody>
</table>

The results show that the both DMF and SolventMix can be used for alkylation adenosine. The yield for all reaction conditions was reasonable, except when DBU was included. The latter base resulted in formation of unspecific products.
Figure 1 $^1$H NMR spectrum collected during alkylation of adenosine at position 1 with compound 4. Specific for the substituted product is the downfield shift of the ribose anomic doublet; 5.92 ppm (unsubstituted adenosine), to 6.08 ppm (product). The spectrum was obtained in deuterated methanol for the sample reacted with alkylation agent over one day at room temperature in SolventMix.

2.2 EFFECT OF pH ON FAD $^1$H NMR SPECTRA AND FAD ALKYLATION

Prior to performing actual FAD modifications, the effect of pH on the $^1$H NMR spectrum of FAD was studied. All $^1$H NMR spectra were collected in D$_2$O at ambient temperature. The pH was adjusted with DCl or NaOD. It can be concluded from Figure 2B that chemical shifts from the adenosine part of FAD are not altered in the pH range 4 – 8. Therefore, alkylation at position 1 can be followed using 1D $^1$H NMR at moderate pH values. Moreover, it was concluded, based on $^1$H NMR analysis, that FAD does not decompose upon microwave irradiation (50 ºC, 50 W). The spectra collected before and after irradiation were virtually identical (data not shown).
**Table 2.** Optimisation results for FAD alkylation at position 1 with allyl halide, NaI and BaCO$_3$ in the SolventMix at 50°C upon microwave irradiation*: A decrease in quality of NMR spectrum was observed upon increase of salt concentration.

<table>
<thead>
<tr>
<th>Time + time with extra additives [h]</th>
<th>extra reagent</th>
<th>final molarity of the reagents</th>
<th>Yield [%] based on $^1$H NMR integrations of signals from sugar anomeric center in reaction mix samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>no</td>
<td>4 (NaI + alkyl halide) + 2 BaCO$_3$</td>
<td>35</td>
</tr>
<tr>
<td>5.5</td>
<td>no</td>
<td>4 (NaI + alkyl halide) + 2 BaCO$_3$</td>
<td>46</td>
</tr>
<tr>
<td>7</td>
<td>no</td>
<td>4 (NaI + alkyl halide) + 2 BaCO$_3$</td>
<td>48</td>
</tr>
<tr>
<td>5.5 + 1.5</td>
<td>NaI + allyl halide</td>
<td>8</td>
<td>57</td>
</tr>
<tr>
<td>5.5 + 3</td>
<td>NaI + allyl halide</td>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td>5.5 + 1.5</td>
<td>BaCO$_3$</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>5.5 + 3</td>
<td>BaCO$_3$</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>5.5 + 4.5</td>
<td>BaCO$_3$</td>
<td>4</td>
<td>59</td>
</tr>
<tr>
<td>7 + 3</td>
<td>BaCO$_3$ + (NaI + allyl halide)</td>
<td>4 + (8)</td>
<td>Over 60 % (± 10%)*</td>
</tr>
</tbody>
</table>

In order to optimize the nucleophilic substitution at the position 1 in FAD with compound 4, several conditions were tested. As tested for adenosine, the effect of adding NaI and BaCO$_3$ was investigated. At the same time, the nucleophilic substitution efficiency of allyl halide over alkyl halide was tested. For that, an alkyl bromide $N$-(4-bromobutyl)-phthalimide was used as an analogue of compound 5.

![Scheme 4.](image)

**Scheme 4.** The alkylation of FAD with alkyl halide was not detectable after 11 days of stirring at 50 °C

$^1$H NMR spectra collected before and after eleven days of stirring did not show any alkylation with the alkyl halide. At the same time, successful alkylation was observed for the allyl halide. Apparently, the lower electrophilicity of the alkyl bromide over the allyl bromide impaired the efficiency of FAD alkylation.

The combined use of NaI and an alkylating agent with stepwise increasing quantities resulted in a dark
red solution with some precipitate and yield improvement (Table 2). Nonetheless, it also triggered difficulties to collect good quality $^1$H NMR spectra. The highest yield of alkylation reached around 60% (Table 2). Noticeably, similar to results for the adenosine $N^6$-alkylation, we obtained a higher reaction yield after stepwise addition of salts and the alkylating agent. Based on the obtained results (Table 1), the following conditions for the $N^6$-substitution of FAD were chosen for further experiments: two times addition of reagents: 2 eqv of alkylating agent, 2 eqv of NaI (4 equivalents) and 1 eqv of BaCO$_3$ at 50 °C, 50 W microwave irradiation.

Figure 2. (A) FAD atom numbering, and (B,C) $^1$H NMR spectra of FAD at different pH values.
2.3 SYNTHESIS OF N1-[4-BUTYL-2-EN]-PHTHALIMIDE]-FAD

For the FAD derivatization on preparative scale, a shorter and more efficient synthesis of another alkylating agent, cis- and trans-N-(4-bromobut-2-en-1-yl)phthalimide (5), was designed and performed as shown in Scheme 5. The only structural difference versus the previously used alkylating agent 4 is the amine protection group. Compound 4 contains the Fmoc group while compound 5 contains phthalimide. Both groups can be cleaved with 5% diethylamine (Fields, 1995; Sen and Roach, 1995). At the same time, due to its strong nucleophilic character, diethylamine can play an important role to promote the Dimroth rearrangement, the next and very last step of the synthesis (section IV). Preparation of compound 5 is simpler and more efficient than the synthesis of 4 (only 2 steps and 32% overall yield versus 4 steps with 7% yield). Details for the synthesis of 5 can be found in the experimental section.

FAD was modified using compound 5 as shown in Scheme 5. Formation of the FAD derivative 6 was observed in the reaction mixture by NMR analysis and LC-MS. Based on the first method, conversion was estimated to be around 60% (Figure 4), which is in line with the with the preliminary optimization experiments in the section 2.2. For purification of compound 6, several approaches were tested including precipitation at high pH, silica gel chromatography, and anion exchange chromatography (Q-Sepharose). The latter approach resulted in separation of several major fractions, which indicates that the compound may degrade when in contact with the column material. Moreover, it was found that this FAD derivative is also not stable upon storage at room temperature. Eventually, the reaction mixture was diluted twice in acidified water (final pH 4) and washed twice with CH2Cl2. Using this procedure, the (unreacted) alkylating agent was removed, which was confirmed by TLC analysis. It also shows that extraction with CH2Cl2 is not harmful for the product.
Scheme 5. The synthesis pathway of \( \text{N}^6\)-(butyl-2-en-4-amine)-FAD. A) alkylating agent synthesis, B) modification at the adenosine position 1 and further rearrangement into the \( \text{N}^6 \) position in combination with phthalimide deprotection.
Synthesis of a Novel Flavin Cofactor Analogue, N6-(Butyl-2-en-4-amine)-FAD.

Figure 3. LC-MS analysis of the reaction mixture of FAD alkylation after two times addition of 4 eq of compound 5 and NaI, and 2 eq of BaCO₃. Total microwaving time in 50 °C and 50 W was 12.5 h. The figure shows a peak in the LC chromatogram (retention time 17.7 min, upper panel) that corresponds to compound 6 (lower panel, double charged: m/z = 439). The peak with retention time 13.5 min corresponds to FAD.

Figure 4. Spectrum of the aqueous phase of compound 6 after purification by two times extraction with CH₂Cl₂. In blue chemical shifts characteristic for the compound derivatized at position 1 (assigned based on the integration), black – peaks from FAD. ¹H NMR (500 MHz, D₂O, pD 4) δ 8.51, 8.36, 8.29, 7.95, 7.78, 7.59, 6.13 (d, J = 5.6 Hz), 5.86 (d, J = 5.0 Hz), 2.54, 2.43, 2.40, 2.34.
**Figure 5.** FAD alkylation with compound 5. $^1$H NMR spectra were collected at room temperature in $\text{D}_2\text{O}$, pD 4. Top: changes in chemical shift of peaks from adenosine C2 and C8 proton singlets; Bottom: doublet from FAD ribose C1’ proton upon alkylation at adenosine position 1. Red – starting reaction mix, green – reaction mix after microwaving and extra reagents addition, blue – aqueous, acidified phase after two times extraction with $\text{CH}_2\text{Cl}_2$. The alkylation agent is insoluble in water. Therefore, a multiplet around 5.8 (double bond) and 7.5 (phthalimide) ppm correspond to the compound 6.

**Table 3.** Chemical shifts for $^1$H NMR signals that do change upon alkylation of adenosine. Data were collected for adenosine, alkylated adenosine in CD$_3$OD and compound 6 in $\text{D}_2\text{O}$.

<table>
<thead>
<tr>
<th>Compound</th>
<th></th>
<th>FAD Proton$^*$</th>
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<tbody>
<tr>
<td></td>
<td>C8</td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td>shift (ppm)</td>
<td>shift (ppm)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>8.24</td>
<td>8.11</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>N’-(4-Fmoc-but-2-en)-adenosine</td>
<td>8.57</td>
<td>8.46</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Compound 6$^c$</td>
<td>8.51</td>
<td>8.36</td>
</tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$: atom numbers as in Figure 6.
$^b$: chemical shift was not assigned because of overlap with another signals.
$^c$: chemical shifts were referred to water peak (4.87 ppm in CD$_3$OD and 4.90 ppm for $\text{D}_2\text{O}$).
2.4 SYNTHESIS OF N^6-(4-AMINE-BUTYL-2-EN)-FAD: DIMROTH REARRANGEMENT AND DEPROTECTION OF N^1-(4-AMINE-BUTYL-2-EN)-FAD

The final, N^6- substituted FAD analogue 7 is obtained from compound 6 via the Dimroth rearrangement and removal of the protecting group, phthalimide. The efficient removal requires a high amount of diethylamine. On the other hand, the pH should remain mild for preserving the FAD molecule. Therefore, two conditions were tested: phosphate buffer pH 8.5 containing 5% and 0.5% of diethylamine. In both cases, deprotection and subsequent Dimroth rearrangement occurred, which was evidenced by formation of compound 7 (monitored by LC-MS). The conditions resulting in higher conversions contained the highest diethylamine concentration. Therefore, for a larger scale conversion, 0.8 M phosphate buffer (pH 8.5) with 5% diethylamine was employed at 50 °C. As this step is relatively fast (Bückmann, Wray and Stocker, 1997) and diethylamine is a volatile compound with a low boiling point, a microwave irradiation was not used. After a few days, the completion of the Dimroth rearrangement was confirmed by LC-MS analysis (Figure).
Table 4. Peaks assignments for N\(^6\)-(butyl-2-en-4-amine)-FAD (compound 7). For atom numbering, see Figure. Assignments are in agreement with literature data (Halada et al., 2003).

<table>
<thead>
<tr>
<th></th>
<th>F7a</th>
<th>F8a</th>
<th>C'3 and C'5</th>
<th>L4</th>
<th>L1</th>
<th>C'5A</th>
<th>C'2B</th>
<th>C'4</th>
<th>C'1A</th>
<th>C'3</th>
<th>C'2</th>
<th>C'4 and C'5B and C'2A</th>
<th>C'1 B</th>
<th>C'1 and L2/L3</th>
<th>F6</th>
<th>F9</th>
<th>C2</th>
<th>C8</th>
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<tr>
<td>H1 [ppm]</td>
<td>2.2</td>
<td>2.29</td>
<td>3.91</td>
<td>3.98</td>
<td>4.09</td>
<td>4.1</td>
<td>4.25</td>
<td>4.29</td>
<td>4.82-4.83</td>
<td>4.5</td>
<td>4.54</td>
<td>4.36-4.37</td>
<td>5</td>
<td>5.82-5.86</td>
<td>7.35</td>
<td>7.44</td>
<td>7.8</td>
<td>8.24</td>
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<tr>
<td>Cross peaks</td>
<td>4.36</td>
<td>5.82-5.85</td>
<td>5.82-5.86</td>
<td>4.29</td>
<td>4.1</td>
<td>3.91</td>
<td>4.36</td>
<td>4.36</td>
<td>5.82</td>
<td>4.5</td>
<td>4.25</td>
<td>4.09</td>
<td>4.64-4.68</td>
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<tr>
<td></td>
<td>4.38</td>
<td>4.36</td>
<td>4.1</td>
<td>4.54</td>
<td>4.5</td>
<td>4.25</td>
<td>4.82</td>
<td>4.54</td>
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* Peaks overlap, not well resolved
The final reaction mixture contained only two main compounds with absorbance in the visual range (LC-MS/Vis detection): FAD (RT = 13.03 min, [ESI-] 785.09 m/z), and a compound with a retention time of 13.13 min (Figure). The UV-Vis spectrum of the latter has typical flavin features with maxima at 265 nm, 371 nm and 446 nm. The absence of a broad absorbance peak at around 300 nm suggests that no significant amount of the tricyclic side, products were formed (van der Plas et al., 1995). The most dominant peak in the mass spectrum has a m/z value of 876, which can be assigned as a sodium adduct of 7. Based on this, no remaining unreacted N\textsuperscript{1}–alkylated FAD, nor tricyclic side products were present after the Dimroth rearrangement reaction. All in all, it can be concluded that the rearrangement yield is close to quantitative as the peak corresponding to compound 6 disappeared, and there are no other peaks with absorbance in the visual range besides some residual FAD.
Figure 7. LC-MS profile of the final Dimroth rearrangement reaction mixture (top graph). The formation of \(N^6\)-FAD is visible at a retention time of 13.13 min; FAD-like spectrum (middle plot), and adduct with sodium ESI + : calculated mass \([M + Na^+] = 877.2\); found 876.66 m/z (bottom plot).

2.5 PURIFICATION OF \(N^6\)-(butyl-2-en-4-amine)-FAD

For the purification of \(N^6\)-(butyl-2-en-4-amine)-FAD, several approaches were tested. The use of anion-exchange material (Q-Sepharose) did not give a good separation and unspecific products were formed (LC-MS analysis). Phenyl-Sepharose gave no separation. The best separation with the least amount of side products was obtained using a C18 column. This purification was followed by a desalting column in order to remove salts. After that, the remaining volatile components (diethylamine) were removed using elevated temperatures and a high vacuum. The purified sample was ninhydrin-positive (TLC analysis, Figure 10). As a negative control, FAD incubated with the same concentration of diethylamine did not give a positive response to a ninhydrin test. This confirms the presence of a primary amine in the final compound. The purity of compound 7 is estimated as 80% based on LC-MS profile (Figure 8). After desalting, the expected mass of compound 7 (856 m/z) was observed by LC-MS (Figure 9).
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**Figure 8.** LC-MS-UV profile of compound 7 after purification using a C18 cartridge (details in experimental section) with retention time 13.28 min. The observed, dominant peak corresponds to the sodium adduct of 7.

**Figure 9.** The mass spectrum in the positive mode. Peak 856 m/z corresponds to a single charged compound 7.

**Figure 10.** TLC analysis of reaction mixture containing compound 7 (lane 1) and diethylamine (lane 2) using ninhydrin for detection. As a negative control, FAD incubated with the same concentration of diethylamine did not give a positive signal (photo not shown).
2.6 CHARACTERISATION OF \(N^6\)-(BUTYL-4-EN-2-AMINE)-FAD

The redox potential of \(7\) was determined by cyclic voltammetry. The voltamperograms obtained for FAD and \(7\) are shown in Figure 11. Both flavins displayed a similar redox behavior and potential (0.205 V vs Ag/AgCl). This is according to expectations, the FAD derivatization at the adenosine \(N^6\) was not expected to alter the flavin redox properties.

![Cyclic voltamperograms](image)

**Figure 11.** Cyclic voltamperograms measured at room temperature in water with 0.1 NaCl and formic acid, pH 3.5 (red: FAD, blue: \(7\)) measured vs AgCl/Cl. Signal from the compound \(7\) contains small redox-active impurity exchange one electrone (around 0.06V difference between oxidation and reduction), and can be assigned to diethylamine (1.14 vs SCE (Novakova et al., 2014)).

For a final check of the prepared material, NMR spectra were obtained. A \(^1\)H spectrum of \(7\) confirmed the presence of an alkene, which was evidenced by a multiplet around 5.87 ppm. This signal overlaps with the doublet from the \(1'\) proton (Figure ). It is in line with HSQC analysis (Fig. S10), which revealed two cross-peaks corresponding to \(^{13}\)C NMR shifts: 88 ppm that corresponds to the \(1'\) moiety.
and 132 ppm corresponding to L2 and L3 atoms (Figure 6). In addition, separation of $^1$H NMR peaks around 5.8 ppm region also was obtained using the COSY technique (Fig. S 9). Considering the fact that the spectra were collected in D$_2$O and compound 5 is not soluble in water, the observed multiplet most probably comes from the linker part of the substituted, water soluble FAD analogue. The overlap of $^1$H NMR spectra of FAD and 7 shows extra signals for the latter one, which correspond with a good agreement to expected chemical shifts for the linker part at the L4 and L1 positions (Figure 6 shows atoms) (4 ppm region, Fig. S8). Moreover, the proton spectrum of 7 indicates that the molecule is fully rearranged into the $N^6$ derivative (Figure 12). This is in line with the absorbance spectrum obtained by LC-MS/UV-Vis analysis, revealing a clear maximum at 267 nm. Moreover, the same peak has a mass corresponding to the adduct with Na$^+$ (Figure 7).
Figure 12. Compound 7 after purification with C18 column. The sample still contains some impurities (grey chemical shifts): diethylamine (peaks 1.26 and 3.05 ppm), compound 6 (multiplet around 7.57 and doublet at 6 ppm). Diastereomeric mixture

The aromatic region of the $^1$H NMR spectrum (Figure 2) has a weak, characteristic multiplet signal originating from the phthalimide, which is also visible in the HSQC spectrum (Fig. S10). Nevertheless, when normalized for the single proton peak from compound 7, the integration is 0.61 which corresponds to around 15% of a phthalimide-protected FAD compound (or less likely: some free phthalimide) left in the sample. It suggests that the deprotection is slower than the Dimroth rearrangement. Difficult to interpret is the 0.30 integration for two singlets, shifted downfield versus the methyl groups of 7 (Figure 3). As in the aromatic region no atypical shifts were detected, and there are no unspecified absorptions in the UV-Vis absorbance spectrum, this might come from unspecific molecules coordinated with diethylamine. Yet, also other FAD modifications may have occurred.

Infrared spectra of $N^6$-(butyl-2-en-4-amine)-FAD and FAD (Fig. S11) indicate the presence of a primary amine. Although most prominent signals from amine N-H stretches (3200 – 3400 cm$^{-1}$) are covered by a broad peak from hydroxyl groups (Fig. S113I), there are specific signals from other spectral regions. A shift in absorption for the N-H bending vibration from 1608 towards smaller wave numbers suggests that the nature of the primary amine has changed (Fig S11 II), possibly due to substitution of the aromatic adenosine N$^6$. Diethylamine was excluded to have such an effect on the spectrum by comparison of the FAD IR-spectrum with the IR-spectrum of FAD incubated with
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diethylamine. Furthermore, in the 820 cm\(^{-1}\) region an extra broad band for compound 7 is observed (Fig S11 III) that can be assigned to N-H wag vibration characteristic for primary amines. Secondary amines have this band at lower frequencies with significantly decreased intensity. Moreover, around 3150 cm\(^{-1}\) the relative intensity is slightly increased, which also belongs to the characteristic of primary amines. Barely observable is the higher intensity of a peak at 808 cm\(^{-1}\), which is described as more intense in aliphatic amines (Fig S11III).

Importantly, it was observed that the \(^1\)H NMR spectrum of N\(^6\)-(butyl-4-en-2-amine)-FAD has changed upon two weeks storage in the dark in water at 4 °C. A new doublet with a chemical shift above 6 ppm and three peaks around 6.60-6.70 ppm appeared, moreover the aromatic region had altered (Figure 3). Literature suggests that, in contrast to the derivatives at adenosine position 1, FAD-N\(^6\) analogues do not require special storage conditions (Bückmann, Wray and Stocker, 1997; El Ashry et al., 2010). Possibly some impurities present in the sample are detrimental for the prepared FAD analogue. Nonetheless, compound 7 in a freeze-dried form and stored in darkness is full preserved for at least three weeks.

![Figure 13. \(^1\)H NMR spectrum of compound 7 after 2 weeks storage in D\(_2\)O in 4 °C in darkness.](image-url)
2.7 APPLICATION OF \(N^6\)-(BUTYL-2-EN-4-AMINE)-FAD FOR THE COFACTOR-MEDIATED IMMOBILIZATION

COVALENT IMMobilIZATION OF \(N^6\)-(BUTYL-2-EN-4-AMINE)-FAD ON SEPHAROSE MATERIAL

Scheme 6. Covalent immobilization of \(N^6\)-(butyl-4-en-2-amine)-FAD on N-hydroxysuccinimidy-Sepharose® via nucleophilic substitution at the terminal primary amine with reactive succinimidyl ester group at the resin. Blue color highlights the natural FAD cofactor moiety. Brown color represents the reactive groups on the commercial resin.

The NHS Sepharose turned yellow after incubation with both \(N^6\)-(butyl-4-en-2-amine)-FAD and FAD. The color remained for both materials after extensive washing with water, a saline solution (2 M NaCl) or with the organic solvents acetonitrile and ethyl acetate. Eventually, 5% diethylamine buffered with saturated KPi buffer pH 8.5 resulted in loss of the yellow color in case of the material incubated with FAD, while the NHS Sepharose incubated with compound 7 remained yellow. It shows that the natural cofactor binds to the NHS Sepharose in a very tight manner and only a combination of highly concentrated salt and a strong amine can cause its release. At the same time, this outcome confirms the robust binding of compound 7 to the Sepharose material (Scheme 6).

COFACTOR-MEDIATED RECONSTITUTION OF PAMO ENZYME ON FAD-SEPHAROSE

The Sepharose material with 7 after incubation with the apo PAMO yielded functional enzyme. This was verified by monitoring the conversion of phenylacetone with GC-MS. In the conversion mixture, accumulation of the enzymatic product (benzyl acetate) was detected only for the material containing \(N^6\)-(butyl-4-en-2-amine)-FAD. The immobilized enzyme was used for two consecutive conversions which revealed that the enzyme retained full activity after repeated usage. Based on the conversion experiment, the calculated active enzyme loading on the Sepharose was very low, around 0.1 mg/mL.
This shows that there is room for improving the immobilization procedure. Nonetheless, the reconstitution of an apo flavoenzyme on the \( \text{N}^6\)-(butyl-4-en-2-amine)-FAD functionalized material was demonstrated (Figure 14).

\[ \text{FAD}\rightarrow\text{NH}_2 \text{ represents } \text{N}^6\text{-}(\text{butyl-4-en-2-amine})\text{-FAD.} \]

**Figure 14** Enzymatic activity of Sepharose containing flavin cofactors after apo-PAMO enzyme reconstitution. It has been tested for the Baeyer-Villiger oxidation of phenylacetone by product detection (GC-MS). A basal amount of benzyl acetate was present in the starting material. FAD-\(\rightarrow\text{NH}_2 \text{ represents } \text{N}^6\text{-}(\text{butyl-4-en-2-amine})\text{-FAD.}

4. CONCLUSIONS

A novel FAD analogue, \( \text{N}^6\text{-}(\text{butyl-4-en-2-amine})\text{-FAD \ has been synthesized starting from FAD. The yield of 40\% was obtained with around 80\% purity. In the first step of the synthetic route, satisfying alklylation at position 1 was achieved within 12.5 \text{ h while using microwave irradiation and a strong electrophile, an allyl halide. The second step, which involved a Dimroth rearrangement, gave close to quantitative yield by using a relatively high concentration of a strong nucleophile, diethylamine. Regarding future applications, one drawback of the presented approach is the difficulty of complete removal of diethylamine from the sample. Nonetheless, the developed synthetic procedure is more effective and less laborious than alternative methods published in the patent and scientific literature (Bückmann, Wray and Stocker, 1997). Additionally, a simple and straightforward synthesis of the alkylating agent \( \text{N}^1\text{-}(\text{butyl-2-en-4-amine})\text{-FAD, which is commercially not available, has been developed (43\% yield). It involves only two steps and uses low-cost and accessible chemicals. The novel FAD analogue could be safely stored in freeze-dried form and it displays identical redox properties when compared with FAD. What is essential, its utility towards the cofactor-mediated
enzyme immobilization has been proven. Furthermore, the newly developed approach towards synthesis of N\textsuperscript{6}-derivatized FAD may also be employed for synthesis of other, similar FAD analogues. The developed method enables synthesis valuable, key chemicals for cofactor-mediated immobilization of flavoenzymes.

5. EXPERIMENTAL SECTION

GENERAL REMARKS

All chemicals and solvents were purchased from Sigma Aldrich or TCI and were used without extra purification step. Work that involved FAD or FAD analogues was performed without direct exposure to light: all the vessels were wrapped in aluminum foil and stored in complete darkness in order to prevent unspecific light-triggered reactions. All NMR spectra of FAD and its derivatives were collected in D\textsubscript{2}O.

For thin layer chromatography (TLC) Merck silica gel 60/Kieselguhr F254 0.25 mm was used. Compounds were visualized using a 365 nm lamp or elemental iodine. Flush chromatography was performed with SiliCycle silica gel type SiliaFlush P60 (230-400 mesh).

MATERIALS

All the chemicals for the synthesis were purchased from Sigma Aldrich or TCI and were used without extra purification. For the immobilization, N-hydroxysuccinimidyl-Sepharose\textsuperscript{®} 4 Fast Flow from Sigma Aldrich was used.

ANALYTICAL METHODS

NMR spectra were recorded on a Varian AMX400 (\textsuperscript{1}H: 400 MHz, \textsuperscript{13}C: 100 MHz) or a Varian Unity Plus (\textsuperscript{1}H: 500 MHz, \textsuperscript{13}C: 125 MHz) spectrometer. Chemical shifts are denoted in \(\delta\)-units (ppm) relative to the residual solvent peak unless another reference is indicated (CDCl\textsubscript{3}: \textsuperscript{1}H \(\delta = 7.26\), \textsuperscript{13}C \(\delta = 77.0\); D\textsubscript{2}O \(\textsuperscript{1}H \delta = 4.79\)). The splitting parameters are named as follows: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet of doublets.

LC-MS was performed using LCQ Fleet Ion Trap LC-MS form Thermo Scientific (Finnigan Surveyor with PDA Plus detector connected to the mass spectrometer with ESI ionisation) with C18 3 \(\mu\)m particles with 100 x 2.1 mm diameters column from GRACE. For the separation solvents A: 0.2 % formic acid in H\textsubscript{2}O, and B: 0.08 % formic acid in acetonitrile were employed with the following programme with the flow rate 0.3 mL/min : 2 min 100 % A, 30 min up to 20 % A, 7 min 20 % A, 1 min up to 100 % A, 12 min 100 % A.
Final product purification was run on an Äkta purifier from Amersham Pharma Biotech using a 40 g Grace™ Reveleris™ SRC C18 Cartridge. Solvents were employed as follow: A) Phosphate buffer pH 4, B) MeOH. 100% A for 12 min, up to 30% A for 5 min, 30% A for 10 min, up to 50 % A for 5 min, 50 % A

FT-IR spectra were recorded on a Perkin Elmer FT-IR spectrometer. For the CV, a µAutolab III potentiostat was used and equipped with a gold disc electrode (working electrode), Ag/AgCl electrode (reference electrode) and a platinum wire (counter electrode).

GC-MS detection of enzymatic conversion of phenylacetone into benzyl acetate was performed on a GC-MS-QP 2010 Ultra from Shimazu with helium as a carrier gas. The instrument was equipped with a non-chiral dimethylsiloxane (100%) column from Agilent (30 mx, 0.25 mm, 0.25 µm). Samples for the GC-MS detection of phenylacetone conversion were prepared by the extraction of the enzymatic conversion mixture solution with two times higher volumes of organic solvent (ethyl acetate).

Injection to GC-MS was performed at 250 °C, the linear flow was 21.7 mL/min and pressure was 34.3 Pa. The column temperature program was as follow: 5 min at 110 °C, ramp up to 180 °C (5 degree per minute) and hold at 180 °C for 4 min. The retention times were 3.78 min for phenylacetone and 4.4 min for benzyl acetate.

5.1 SYNTHESSES

2-(4-HYDROXYBUT-2-EN-1-YL)PHTALAMIDE-1,3-DIONE (1)

Compound 1 was obtained using a published procedure (Al-Shuhaib et al., 2013). Briefly, a Mitsunobu reaction was performed in 400 mL of dry THF under oxygen free conditions. To the flask on the ice bath, 15 g isomeric mixture of 1,4-butenediol (170 mmol), an equimolar amount of phthalimide (25 g), and 1.2 molar equivalents (53 g, 204 mmol) of PPh3 were added. Cold 40% DEAD in toluene and 4 mL of dry THF was added in 1.1 molar equivalent (74 g, 187 mmol) dropwise within 15 min. After that, the ice bath was removed and the reaction was continued at room temperature over 3 days. Progress of the reaction was followed on TCL using a EtOAc : pentane (7 : 3 v/v) mixture (Rftrans = 0.35; Rfcis = 0.27). Purification was performed on a silica column with the same combination of solvents. The desired product 1 was obtained in 46% yield as a white solid. Cis and trans isomers were mixed and used for further reactions.

Below are NMR spectra of the isolated trans isomer with detectable signals from the cis isomer. For that reason the integration of alkene’s protons has slightly decreased accuracy.

$^1$H NMR (400 MHz, cdcl3) δ 7.85 (s, ), 7.72, , 5.84 (m, 2H) 4.39 (d, J = 4 Hz, 2H), 4.30 (d, J = 4 Hz 2H), 1.55 (s, OH).

$^{13}$C NMR (100 MHz, cdcl3) δ 163.0, 134.0, 133.0, 131.9, 124.7, 123.2, 62.4, 39.3
The analytical data are in agreement with a previous study (Al-Shuhaib et al., 2013).
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Fig. S1 $^1$H NMR spectrum of trans form compound 1

Fig. S2 $^{13}$C NMR spectrum of compound 1

4-AMINOBUT-2-ENE-1-OL (2) AND 4-(FMOC-AMINO)-1-BUT-2-ENE-OL (3)
Compound 1 (15 g, 69 mmol) was deprotected from phthalimide using 1.1 equivalent of hydrazine hydrate (2.43 g, 76 mmol) dissolved in ethanol overnight at reflux. The reaction was monitored by TLC using EtOAc : pentane 1:1 (v/v) as a solvent mix. The reaction mixture was filtered over a glass filter with cold ethanol three times in order to remove side products. Ethanol was evaporated on a rotary evaporator and white crystalline material has been obtained. It was reacted with Fmoc-Cl as previously reported (Marsault et al., 2006). Briefly, 34.5 mmol of 2 (2.15 g) together with 1.1 equivalent of Fmoc-Cl (7 g, 38.0 mmol) were stirred in 150 mL acetonitrile for 3 hours at room temperature. The TLC with pentane and EtOAc 4:1 (v/v) analysis confirmed completion of the reaction. Acetonitrile was evaporated and the sample was purified with flash chromatography using the same solvent mix. After evaporation, a white solid was obtained with 19% yield after two steps.

\(^1\)H NMR (400 MHz, cdcl\(_3\)) δ 7.74 (d, J = 7.5 Hz, 2H), 7.56 (d, J = 7.3 Hz, 2H), 7.42 – 7.24 (m, 4H), 5.75-5.72 (m, 1.5 H), 4.95 (s, broad OH), 4.55 (d, 2H), 4.40 (d, J = 7.0 Hz, 2H), 4.20 (d, J = 6.8 Hz, 1H), 4.12 (s, 1.5 H), 3.81 (s, 1.5 H), 2 (s, broad)

\(^1^3\)C NMR (101 MHz, cdcl\(_3\)) δ 156.68, 144.13, 141.53, 131.35, 127.93, 127.29, 125.23, 120.22, 66.87, 62.83, 47.46, 42.56.

The analytical data are in agreement with Marsault et al. (2006). The 1.5 H integrations can be the result of having two forms, cis and trans, for which those shifts are different. The dominant isomer is cis (> 75%).
Synthesis of a Novel Flavin Cofactor Analogue, N6-(Butyl-2-en-4-amine)-FAD,…

Compound 3 was brominated as described before (Corey and Kim, 1972). In a Schlenk flask on ice 0.53 g (1.8 mmol) of compound 3 was mixed in 100 mL of dry dichloromethane with PPh₃ (0.52 g, 2.01 mmol, 1.12 eq). Subsequently, 2 mL of cool dry dichloromethane with CBr₄ (0.66 g, 2.01 mmol) solution was added dropwise under inert conditions. A milky solution turned into clear lemon coloured liquid. After addition, the ice bath was removed and the reaction was stirred overnight. TLC with EtOAc and pentane 7:3 (v/v) confirmed that the reaction was complete (Rᵢ = 0.85; Rᵢ = 0.80 for cis and trans). The material was filtered over a glass filter and purified on a silica gel column. After solvent evaporation the product was obtained as a white crystalline solid with 86% yield.

¹H NMR (400 MHz, cdcl₃) δ 7.74 (d, J = 7.6 Hz, 2H), 7.56 (d, J = 7.5 Hz, 2H), 7.34 (m, J = 34.8, 7.5 Hz, 4H), 5.74-5.78 (m, 1.2 H*), 4.80 (m, 0.8H*), 4.42 (d, J = 7.0 Hz, 2H) 4.19 (s, 1H), 3.91 (d, J = 5.9 Hz, 2H), 3.81 (s, 2H), *non-integer integration due to the diastereomeric mixture

¹³C NMR (101 MHz, cdcl₃) δ 156.45, 144.13, 141.60, 131.77, 127.98, 127.32, 125.25, 120.27, 66.95, 47.53, 42.22, 32.12.
Fig. S4. $^1$H NMR spectrum of compound 4

Fig. S5 $^{13}$C NMR spectrum of compound 4
The confirmation that bromination occurred comes from inspecting the $^{13}$C NMR spectra that reveals a significant upfield chemical shift for the carbon directly attached to bromide from 62.83 ppm into 32.12 ppm.

$N$-(4-BROMOBUT-2-EN-1-YL)PHTHALIMIDE (5)

Compound 5 was prepared in the same way as compound 4, only solvent for TLC ($R_f = 0.78$) and flash chromatography was different, using dichloromethane. Bromination was again confirmed with $^{13}$C NMR (Fig. S7). Fig. S6 shows the NMR spectrum of both combined isomers (cis:trans ratio 1:2.5).

CIS: $^1$H NMR (400 MHz, cdcl3) $\delta$ 7.76 – 7.61 (m, 4H), 5.96 – 5.73 (m, 2H), 4.33 – 4.24 (dd, 1H), 3.85 (dd, 1H). TRANS  $^1$H NMR (400 MHz, cdcl3) $\delta$ 7.78 – 7.60 (m, 4H), 5.66 – 5.52 (m, 2H), 4.28 (dd, $J = 4.9$, 3.6 Hz, 2H), 4.12 (dd, $J = 8.2$, 3.8 Hz, 2H).

$^{13}$C NMR (101 MHz, cdcl3) $\delta$ 156.7, 134.55, 130.43, 128.81, 123.86, 120.35, 39.08, 31.72

$N^\text{4'}$-(BUTYL-2-EN-4-AMINE)-FAD (6)

FAD (0.56 g, 0.71 mmol) was dissolved in water and 4 molar eq (2.87 mmol) of each: 5 (0.8 g), NaI (0.42 g), BaCO$_3$ (0.51 g) was dissolved in DMF and DMSO. Solutions were combined so that the final composition of a solvent gave DMSO : DMF : H$_2$O (2:1:1 v/v/v). The reaction mixture was stirred at 50 °C, and another 4 molar equivalents of alkylating reagents was added. After a total microwaving time equal to 12.5 h (50 °C, 50 W) the reaction mixture was diluted 2 times with acidified water (final pH 4) and subsequently twice extracted with CH$_2$Cl$_2$. Subsequently, evaporation was performed using a rotary evaporator under reduced pressure in order to remove trace amounts of organic solvents. After these procedures, the compound 5 was not detectable by TLC analysis (TLC was performed using MeOH:acetone:ethyl ether, 4:1:1 v/v/v). Compound 6 was obtained in the reaction mixture as measured by 1H NMR the most important peaks could be assigned (4).

$N^\text{4'}$-(BUTYL-2-EN-4-AMINE)-FAD (7)

An aquatic solution from the previous step was appropriately diluted with a stock potassium phosphate buffer solution and subsequently with diethylamine. The final solution had a pH of 8.5, contained 0.4 M phosphate and 5 % of diethylamine. The reaction was stirred at 50 °C on an oil bath until the complete rearrangement of 6 into 7 (monitored by LC-MS).

$^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.24 (s, 1H), 7.80 (s, 1H), 7.46 (s, 1H), 7.36 (s, 1H), 5.87 – 5.84 (m, 2H), 5.83 (d, $J = 5.2$ Hz, 1H), 4.78 – 4.61 (m, 1H), 4.51 (dt, $J = 24.2$, 4.9 Hz, 2H), 4.39 – 4.27 (m, 7H), 4.08-3.95 (m, 5H), 3.92-3.87 (m, 1H), 2.29 (s, 3H), 2.20 (s, 3H)

HRMS ESI + : calculated mass [M + H+] = 854.22; found 854.22
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Fig. S6 $^1$H NMR spectrum of compound 5

Fig. S7 $^{13}$C NMR spectra of compound 4 (upper) and 5 (lower)
A comparison of $^1$H NMR spectra of FAD and 7 collected in the same pD (6.5) is shown in Fig. S8.

**Fig. S8** Comparison of $^1$H NMR spectra of FAD (green) and $N^6$-(butyl-2-en-4-amine)-FAD, compound 7 (red). Both were collected in D$_2$O, pD 6.5.
A)
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**Fig. S 9.** COSY spectrum of compound 7: A- full spectrum, B-magnification
Fig. S10 HSQC spectrum of compound 7
IR spectra

Fig. S11. IR spectra of solid compounds. Yellow – FAD, Black – compound 3. Spectra I show full range comparison. Spectra II and III are zoomed for primary amine specific regions.

5.2 ANALYTICAL DATA COLLECTED DURING OPTIMIZATION OF ADENOSINE ALKYLATION

ADENOSINE $N^3$ SUBSTITUTION DETECTION WITH NMR

All the spectra were collected in MeOD. Chemical shifts were adjusted to the water peak (4.78 in methanol) as it had very high intensity in each sample. The coupling constants $J(1'\cdot2')$ for analyzed doublets assigned to position 1 and $N^6$ analogues are in agreement with the literature data (van der Plas, Henk C. Bückmann and Wray, 1995).
Alkylation in RT: (blue) 2 days in DMF; (green) 2 days in DMF:DMSO:H2O; (red) 4 days in DMF:DMSO:H2O

Fig. S12. $^1$H NMR (400 MHz, spectrum was collected in methanol-d$_4$ (reaction in DMF), or D$_2$O. The typical J values for peak around 6.1 ppm was 5 Hz, and for peak 6.0 ppm 6 Hz. Upper spectra show alkylation at room temperature, lower spectrum shows alkylation upon microwave irradiation (50 W)
Chapter 3

5.3 Preparation of FAD-Functionalized Sepharose and Immobilization of Phenylacetone Monooxygenase (PAMO)

Aliquots of 60 µl of Sepharose were washed three times with distilled water and incubated with 2 mL of around 10 mM of compound 7, FAD or MQ water at 4°C, overnight. All but the latter sample turned yellow and retained this color upon washing with ethyl acetate, saturated NaCl solution and aquatic solutions at pH 7, 4 and 8. Subsequently, 5% diethylamine in 1.5 M KPi buffer pH 8 was used for the final wash and blocking of remaining active groups at the agarose. In contrast to sample incubated with 7, the material incubated with FAD, almost completely lost its yellow color after the final wash with diethylamine. Subsequently, the materials were equilibrated with 50 mM Tris pH 8 and incubated with apo PAMO prepared as reported previously (Krzek et al., 2016). To remove remaining apo PAMO, material was washed with buffer three times by spinning down and resuspension. To test enzyme activity, the resulting material was incubated with phenylacetone as it has been described before (Krzek et al., 2016). Two time point samples were analyzed by GC-MS, after 7 hours of conversion.

References


