Kinetics and inhibition of enzymes in early stage drug discovery
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Chapter 3

Enzyme kinetics and inhibition of histone acetyltransferase KAT8

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Introduction

Epigenetic modifications of histones, such as a lysine acetylation, play a key role in the regulation of gene transcription. Histone acetyltransferases (HATs) are a class of acetyltransferases that catalyze the acetylation of ε-amino groups on lysine residues in both histone and non-histone proteins. Through histone acetylation, they play a regulatory role in the chromatin structure, thereby influencing gene transcription. The acetylation of non-histone proteins, for example transcription factors, is involved in the regulation of many processes, such as cell growth and inflammatory signaling (1). This enzyme class has been linked to the pathology of various diseases, including cancer (2-4), inflammatory diseases (5-7), viral infections (8) and neurological diseases (9,10). However, knowledge on their role in specific diseases and drug discovery efforts towards this class of enzymes are still limited. In particular the HAT lysine acetyltransferase 8 (KAT8) is marginally explored in drug discovery projects.

The HATs are a disparate group of enzymes that can be divided into different families based on their structural homology. The three main families are GNAT (Gcn5-related N-acetyltransferases), MYST (MOZ, YBF2/SAS3, SAS2 and TIP60) and p300/CBP (CREB binding protein). The HAT of our interest, KAT8, is a member of the MYST family. This enzyme was originally discovered in Drosophila, where it is involved in dose-compensation of the X-chromosome gene transcription in male flies. KAT8 functions in two protein complexes, MSL and MSL1v1, that are conserved throughout the eukaryotic kingdom, including humans(11). Both KAT8 complexes have been described to be responsible for acetylating lysine 16 on histone H4 (H4K16) and were shown to play a role in cell cycle progression (12). However, only the MSL1v1 complex seems to be involved in acetylation of the tumor suppressor protein p53 (13). KAT8 has also been shown to play a role in embryonic stem cell renewal. Embryonic stem cells lacking KAT8 lose differentiation potential and show changes in morphology and gene expression of essential transcription factors (14). Thus, it is clear that KAT8 plays a very important role in normal physiology and disease.

KAT8 is a bi-substrate enzyme that binds two substrates; acetyl coenzyme A (Ac-CoA) and histone H4 containing free lysine ε-amino groups. Development of inhibitors for bi-substrate enzymes requires knowledge of the catalytic mechanism. It is important to understand if the substrates, acetyl coenzyme A and histone H4, bind simultaneously or consequently and if the individual binding events are inter-dependent. In addition, knowledge on the catalytic mechanism combined with inhibitor kinetics, enables the calculation of the assay-independent inhibition constant ($K_i$) from the assay-dependent inhibitory concentration ($IC_{50}$) as described by Cheng and Prusoff (15). Therefore, we investigated the catalytic mechanism of KAT8 using enzyme kinetic studies based on models described by Copeland (16). We demonstrated that the non-selective HAT inhibitor anacardic acid (AA) (17) also inhibits KAT8 and performed kinetic studies to further investigate this inhibitor. Based on the results, we proposed a model comprising the catalytic activity of KAT8 and the inhibitory action of AA. We employed this
knowledge to study the inhibitory potency of a small collection of anacardic acid derived inhibitors and to calculate their respective binding constants ($K_i$). Inhibition studies on p300 did not reveal selectivity between both enzymes for the compound collection that was investigated.

**Results and discussion**

**Catalytic mechanism**

As described by Copeland (16), steady state kinetic experiments can be employed to determine by which catalytic mechanism the enzyme operates. In our studies we adapted an enzyme activity assay based on fluorescence detection of the HAT reaction product CoA, as described by Gao et al. (18) for use with KAT8. In this assay the CoA thiolate is detected by the thiol sensitive fluorescent dye 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM). As histone substrate, a synthetic peptide corresponding to the first 20 amino acids of the histone H4 N-terminal (histone H4 peptide) was used. First, the $K_m$ of the histone substrate and $k_{cat}$ of the enzyme were determined using increasing concentrations of histone substrate at constant concentration of Ac-CoA (Figure 1A). Then the catalytic mechanism was determined according to procedures described by Copeland. The velocity of recombinantly expressed KAT8 was determined at different concentrations of Ac-CoA in the presence of varying concentrations of histone substrate (Figure 1B). The $K_m$ and $V_{max}$ of Ac-CoA were determined. Both $K_m$ and $V_{max}$ of Ac-CoA increased at increasing concentrations of histone substrate (Table 1).

![Figure 1: Kinetics and catalytic mechanism of the bi-substrate enzyme KAT8. A) Determination of $V_{max}$, $K_m$ and $k_{cat}$ of the histone substrate (histone H4 peptide). The steady-state velocity was determined of recombinantly expressed KAT8 using 0 - 400 µM histone substrate and 4µM Ac-CoA. $V_{max}$, $K_m$ and $k_{cat}$ of the histone substrate were derived from the non-linear Michaelis-Menten regression. B) KAT8 operates via a ping-pong mechanism. The steady-state velocity was determined of KAT8 at 0 – 20 µM of Ac-CoA in the presence of 15, 30, 60 and 90 µM of histone substrate. Both $K_m$ and $V_{max}$ of Ac-CoA increased at increasing concentrations of histone substrate (H4peptide), which suggests a ping-pong mechanism.](image)

<table>
<thead>
<tr>
<th>Histone H4 peptide</th>
<th>$V_{max}$ (pmol/min)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15µM</td>
<td>3.4 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>30µM</td>
<td>4.5 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>60µM</td>
<td>9.9 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>0.7 ± 0.04</td>
</tr>
<tr>
<td>90µM</td>
<td>14.7 ± 0.6</td>
<td>2.8 ± 0.5</td>
<td>1.1 ± 0.06</td>
</tr>
</tbody>
</table>
which is characteristic for a ping-pong mechanism. In a ping-pong mechanism the donor substrate binds first to the enzyme. In case of KAT8, Ac-CoA acts as an acetyl donor. Subsequently the acetyl group is transferred temporarily to a residue on the enzyme and CoA leaves the binding pocket. Then the histone substrate binds and the acetyl group is transferred onto its lysine residue, upon which the second product is formed.

From the $V_{\text{max}}$ and the enzyme concentration, the turnover number of the enzyme ($k_{\text{cat}}$) can be calculated. This is the maximum number of substrate molecules that the enzyme can convert per catalytic site per unit of time. The $k_{\text{cat}}$ ranged between 0.2 and 1.1 molecules per minute, which is lower than the $k_{\text{cat}}$ observed by Yang et al. (19). This difference can be explained by the concentration dependency of $V_{\text{max}}$ and $k_{\text{cat}}$ on the concentration of both substrates (Table 1). As described by Copeland, in case of a ping-pong mechanism the histone peptide should have little or no affinity for the enzyme in absence of Ac-CoA. To find further evidence for this mechanism isothermal titration

**Figure 2:** ITC experiments support a ping-pong mechanism. ITC experiments were done to determine the affinity of the substrates for KAT8. Ac-CoA had a $K_d$ of 8.7 µM for KAT8. The histone substrate (histone H4 peptide) had a $K_d$ of 6.7 µM for the KAT8/Ac-CoA complex, but did not show affinity for KAT8 without Ac-CoA present. This supports a ping-pong mechanism. The binding energies were calculated and revealed that binding of both Ac-CoA and histone H4 peptide was due to an increase in entropy.

**Table 2:** Parameters for Ac-CoA and the histone substrate as derived from ITC experiments (Figure 2).

<table>
<thead>
<tr>
<th></th>
<th>Ac-CoA vs KAT8</th>
<th>Histone H4 peptide vs Ac-CoA-KAT8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (µM)</td>
<td>$8.7 \pm 1.2$</td>
<td>$6.7 \pm 2.1$</td>
</tr>
<tr>
<td>$N$ (stoichiometry)</td>
<td>0.95</td>
<td>1.03</td>
</tr>
<tr>
<td>$\Delta H$ (cal/mol)</td>
<td>$10470 \pm 470$</td>
<td>$3732 \pm 300$</td>
</tr>
<tr>
<td>$-T\Delta S$ (cal/mol/degK)</td>
<td>$-17228$</td>
<td>$-10665$</td>
</tr>
<tr>
<td>$\Delta G$</td>
<td>$-6758$</td>
<td>$-6933$</td>
</tr>
</tbody>
</table>
Enzyme kinetics and inhibition of histone acetyltransferase KAT8

calorimetry (ITC) experiments were performed (Figure 2). Titration of Ac-CoA to KAT8 indicated binding with an equilibrium dissociation binding constant ($K_d$) of 8.7 µM (Table 2). This is close to the $K_d$ determined for GNAT family HATs (20) and the yeast HAT ESA1 in the picNuA4 complex (21). The stoichiometry (N) of the interaction was close to 1, showing that one molecule of Ac-CoA binds to one molecule of enzyme. Subsequent titration of histone substrate to the KAT8/Ac-CoA complex demonstrated a $K_d$ of 6.7 µM for the histone substrate. In contrast, titration of the histone substrate solution to the KAT8 enzyme in absence of Ac-CoA showed no measurable binding. These observations are consistent with the ping-pong mechanism for the KAT8 acetyltransferase activity as postulated based on the enzyme kinetic experiments, providing further proof for this mechanism.

It is of interest to mention that binding of both substrates has an unfavorable enthalpy component that is compensated for by a strongly favorable entropy component (Table 2). Strongly favorable entropy components in equilibrium binding kinetics are often linked to the release of bound water molecules upon substrate binding, which may also be the case in KAT8. A requirement for a ping-pong mechanism is that the binding pocket has a residue that can temporarily accept the acetyl group before transfer to the substrate. This role is usually taken by a cysteine residue because acetylation of a cysteine residue results in a thioester, which is prone to aminolysis by the lysine ε-amine functionalities of the histone substrates. Previously published KAT8 crystal structures (PDB: 2GIV, 3TOA (19)) indicate that cysteine-143 is close to the binding site of Ac-CoA (Figure 3A). It seems reasonable to presume that this cysteine residue plays a role as an initial acceptor of the acetyl group in the postulated ping-pong mechanism, although none of these published crystals structures contain an acetylated cysteine residue in this position. Surprisingly, close to this cysteine, both crystal structures show an acetylated lysine residue (Ac-Lys-101) that is auto-acetylated by KAT8 itself. As shown by mutagenesis studies, both the cysteine and lysine residues were essential for HAT activity (19). The observation that the cysteine residue is essential for catalysis also supports the postulated ping-pong mechanism.

Further support for the ping-pong mechanism in KAT8 catalysis comes from the crystal structure of the yeast HAT ESA1, which has a high sequence and structural similarity with KAT8. Importantly, the Ac-CoA binding pocket is almost identical and the aforementioned cysteine and lysine residues are conserved (Cys-304 and Lys-262). Interestingly, a crystal structure of ESA1 co-crystallized with Ac-CoA showed bound CoA rather than Ac-CoA and acetylation of cysteine-304 (PDB: 1MJA, Figure 3B) (22). Taken together, the postulated ping-pong mechanism as observed in the enzyme kinetic experiments can be rationalized by structural data for KAT8 and closely related HATs thus further supporting the evidence for this mechanism.

Despite this evidence we should note that enzyme kinetics for HATs frequently seem to depend on the assay conditions. For example, in contrast to the structural data described before, a kinetic study on the yeast analog ESA1 indicated the formation of a ternary complex between the enzyme, Ac-CoA and the substrate in catalysis (21).
Comparable complications were encountered in the analysis of the catalytic mechanism of p300. Based on enzyme kinetics a ping-pong mechanism was proposed (23). However, it was demonstrated that an electrophilic acetyl-CoA affinity labelling-based probe did not target a residue that is critical for catalysis, arguing against a ping-pong mechanism (24), but a ternary complex mechanism could not be confirmed either. Despite the fact that p300 contains a cysteine in the binding pocket no mutagenesis studies have yet been performed to investigate whether this cysteine is important for HAT activity. Based on structural and biochemical data, it was proposed that p300/CBP uses a modified mechanism denoted as the Theorell-Chance (‘hit-and-run’) catalytic mechanism. In the Theorell–Chance mechanism, there is no stable ternary complex as formed in a standard ternary complex mechanism. After acetyl-CoA binds, the histone substrate associates weakly with the p300 surface, allowing the lysine to react with the acetyl group, but kinetically only the interaction with acetyl-CoA is important (25). Therefore, we do not exclude the possibility that a more refined enzyme kinetic model will be assigned to KAT8 in the future. Nevertheless, the steady state kinetic study here clearly indicates a ping-pong mechanism in which Ac-CoA binds first followed by binding of the histone H4 peptide. Therefore, we applied this mechanism for calculation of the $K_i$ values of inhibitors of this enzyme using an adaptation of the Cheng-Prusoff equation as described by Copeland (16).

**Inhibitors – Chemistry**

Anacardic acid is a known natural product HAT inhibitor, which also shows activity on KAT8. A focused compound collection inspired by AA was assembled from newly synthesized compounds 8a-b, 11c-e and 13, previously synthesized compounds 14 and

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**Figure 3:** Structural support for a ping-pong mechanism. A) A crystal structure of KAT8 (PDB:2GIV) shows Ac-CoA bound to the enzyme. Close to the binding pocket, a cysteine is situated, which could facilitate a ping-pong mechanism. Additionally, an acetylated lysine is shown, which is essential for activity of KAT8 (19). B) A crystal structure of yeast ESA1, a MYST family HAT and homologue to KAT5, shows an almost identical binding pocket with an acetylated cysteine. It is possible that either the cysteine or the lysine, or both, contribute to a ping-pong mechanism.
Enzyme kinetics and inhibition of histone acetyltransferase KAT8

15 (26) and the known p300 inhibitor C646. The compound collection was designed to vary the position of alkylation of the salicylate core and the length and polarity of the aliphatic substituent. The compounds were synthesized, using a convenient and flexible synthetic route employing Sonogashira coupling as a key step as published previously (27). Using this strategy, different salicylate triflates or halides were linked to various alkynes.

6-alkyl substitution of the salicylate core was achieved using Sonogashira coupling of terminal alkynes to triflate 5. Triflate 5 was synthesized from 2,6-dihydroxybenzoic acid using a previously published two-step synthesis (28). 5-alkyl substitution of the salicylate core was achieved with aryl halides 9 as starting materials, which were prepared using

6. Scheme 1: Synthesis of anacardic acid derivatives. a) lithium hydroxide, THF/water, RT; b) LiAlH$_4$, THF, 0 °C - RT; c) Benzyl bromide, NaH, THF, 0 °C - RT; d) PdCl$_2$(PPh$_3$)$_2$, Cul, Et$_3$NH, CH$_3$CN, respective aliphatic alkynes or alkyne 4a-b, 100 °C, MW; e) Hydrogenation triple bond and hydrogenolysis of benzyl protected salicylic acids or alkynes: H, (3 atm), Pd/C, MeOH/ethyl acetate, 40 °C, PARR apparatus; f) 5N KOH, THF, 55 °C or HCl (37%), dioxane, RT; g) deprotection of methyl protected salicylic acids: 1M BBr$_3$ in DCM, acetonitrile, -78 °C to RT; h) 1-heptanethiol, KOH, MeOH, 0 °C to RT.
known procedures (29,30). Commercially available terminal alkynes were used, except for terminal alkynes 4a and 4b. Alkyne 4a was synthesized by benzylation of 3a using previously published procedures (31) (Scheme 1A). Alkyne 4b was synthesized from methyl undec-10-ynoate (1) by hydrolysis of the ester (2), reduction of the acid (3b) and subsequent benzylation in an overall yield of 44% over 3 steps. Sonogashira couplings in the salicylate 6-position (Scheme 1B) and the 5-position (Scheme 1C) were performed with moderate to good yields (46-98%). The purification was in some cases demanding due to side products formed in the reaction. The resulting alkynes were reduced by hydrogenation, which simultaneously resulted in the removal of the benzyl moiety from the aliphatic alcohols. Intermediate 6b could not be completely purified after the Sonogashira coupling due to presence of a side product with similar polarity. After an initial hydrogenation step, which proved to reduce the triple bond, the impurity could be removed. Removal of the benzyl required a separate hydrogenolysis step giving 7b in 60% yield. Hydrolysis of the acetonide of 7a using the published procedures of 5N KOH in THF/water at 55°C required 5 days giving 8a in 24% yield. Therefore 7b was deprotected by acid using 11 M HCl in dioxane/water. An improved yield of 46% was observed under these conditions. The methyl protecting group of 10c was removed with boron tribromide in 53% yield. The removal of the benzyl protective group could be done simultaneously with the hydrogenation and was therefore used for the other compounds. Hydrogenation and hydrogenolysis of 10d-e was done very efficiently, giving 11d-e in 88-97% yield. To investigate whether the salicylic acid could be replaced by a 2-hydroxy acetophenone group, product 13 was synthesized (Scheme 1D). A substitution reaction using 2-bromo-2′-hydroxyacetophenone 12 and 1-heptane thiol provided 13 in one step in 85% yield.

Inhibitor kinetics-KAT8
To investigate the binding kinetics of the inhibitors, we conducted enzyme kinetic studies on KAT8 with AA. The velocity of KAT8 was determined at different concentrations of Ac-CoA and constant concentration of the histone substrate in the presence of varying concentrations of AA (Figure 4A). A clear decrease in $V_{\text{max}}$ and $K_m$ with increasing concentrations of AA was observed (Table 3). The decrease in $V_{\text{max}}$ indicates that AA binds a site allosteric from the Ac-CoA binding pocket. The decrease in $K_m$ suggests that the binding of AA stabilizes the binding of Ac-CoA. This is a characteristic of uncompetitive inhibition, where the substrate must be present for the inhibitor to bind. The same experiment was done using different concentrations of the histone substrate and a constant concentration of Ac-CoA (Figure 4B). Strikingly, the curves do not follow Michaelis-Menten kinetics when AA is present, but have a sigmoidal appearance. This shows that there is cooperativity resulting in a Hill coefficient, a measure for the slope of the curve, that is not equal to 1 as described in the Monod-Wyman-Changeux (MWC) model (32). It is not possible to derive a true $K_m$ from a sigmoidal curve and therefore the concentration of peptide that gives half-maximal velocity (khalf) is determined, which resembles the $K_m$, but is dependent on the hill slope (for calculation of khalf see SI). At increasing concentrations of AA, the $V_{\text{max}}$ is constant, but an increase is observed in khalf. This suggests that the binding of AA opposes the binding of the histone substrate.

The cooperativity may be explained using a model based on the existence of two
Enzyme kinetics and inhibition of histone acetyltransferase KAT8

Figure 4: Inhibitor kinetics of AA on KAT8. The velocity of KAT8 was determined at 0 – 20 µM Ac-CoA in the presence of 0, 80, 160 and 240 µM AA respectively. Both $K_m$ and $V_{max}$ decrease at increasing concentrations of AA. The velocity of KAT8 was determined at 0 – 200 µM of the histone substrate (histone H4 peptide) in the presence of 0, 80, 160 and 240 µM AA respectively.

Table 3: $V_{max}$ and $K_m$ for Ac-CoA at different concentrations of inhibitor AA. $V_{max}$, hill slope and $k_{half}$ for histone H4 peptide at different concentrations of inhibitor AA (Figure 4).

<table>
<thead>
<tr>
<th>Ac-CoA [AA] (µM)</th>
<th>$V_{max}$ (pmol/min)</th>
<th>$K_m$ (µM)</th>
<th>histone H4 peptide $V_{max}$ (pmol/min)</th>
<th>Hill slope</th>
<th>$k_{half}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17 ± 0.7</td>
<td>4.8 ± 0.5</td>
<td>13 ± 0.7</td>
<td>1.5 ± 0.2</td>
<td>36</td>
</tr>
<tr>
<td>80</td>
<td>12 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>12 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td>47</td>
</tr>
<tr>
<td>160</td>
<td>9.2 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td>12 ± 0.5</td>
<td>3.4 ± 0.6</td>
<td>54</td>
</tr>
<tr>
<td>240</td>
<td>6.1 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>12 ± 0.4</td>
<td>3.4 ± 0.3</td>
<td>514</td>
</tr>
</tbody>
</table>

conformations of the enzyme, E and E* (Figure 5). Ac-CoA binds the free enzyme (E), which has low catalytic activity. The histone substrate is not able to bind this conformation, which is shown by the ITC data (Figure 2). Upon binding of Ac-CoA, the acetyl group (X) is transferred onto the enzyme (EX) and the histone substrate (S) can bind. This is shown by the ITC experiments as well as the mechanistic studies. Binding of the histone substrate induces a conformational change (E*XS), which is catalytically active. Ac-CoA has lower or no affinity for this conformation, which is shown by the increase in $K_m$ of Ac-CoA induced by higher concentrations of histone H4 peptide (Figure 1). The histone substrate is acetylated, leaves the enzyme as product (Ac-histone substrate) and free enzyme (E) is regenerated. The inhibitor AA binds to EX, thereby stabilizing the catalytically inactive conformation of the enzyme (E). This is shown by the increase in $K_m$ of the histone substrate and especially by the decrease in $K_m$ of Ac-CoA. Ac-CoA regains affinity for the enzyme, even though the enzyme activity is inhibited. Additionally, an increasing concentration of histone substrate, which induces conformation E* for which AA has little or no affinity, will eventually be able to restore activity of the enzyme. Cooperativity between an active and an inactive conformation can cause the sigmoidal behavior observed in the inhibitor kinetics with the histone substrate. This was also observed in case of the bi-substrate enzyme phosphofructokinase. Michaelis-Menten kinetics can be observed when the enzyme has maximal activity. A shift towards sigmoidal kinetics is therefore only observed in case of the histone substrate where the $k_{half}$ decreases due to the presence of an inhibitor (33). Based on mechanistic and inhibitor kinetic studies, we propose a model comprising the catalytic activity of KAT8 and the inhibitory action of AA, which can be used to further investigate the inhibitor properties.
**Figure 5:** Proposed model for the inhibitory and catalytic activity of AA and KAT8. Ac-CoA binds to the free enzyme (E), which has low catalytic activity. The histone substrate is not able to bind this conformation. Following the ping-pong mechanism, the acetyl group (X) is transferred to the enzyme (EX). Binding of the histone substrate (S) induces a conformational change of the enzyme (E*XS), which is catalytically active. Ac-CoA has no or lower affinity for this conformation. The histone substrate is acetylated and leaves the enzyme as product (Ac-histone substrate) upon which the free enzyme conformation (E) is regenerated. The inhibitor AA binds to EX and stabilizes the catalytically inactive conformation, therefore inhibiting the catalytic activity of the enzyme, but increasing the affinity of Ac-CoA.

**Inhibitors – Kᵢ calculation**

The inhibitory potency for the HAT enzyme KAT8 was determined using the same fluorescence-based assay as used for the kinetic studies. The inhibitory concentrations 50% (IC₅₀) were determined if more than 50% inhibition was observed at 400 µM inhibitor concentration. As shown in the kinetic studies for the catalytic mechanism, the Kᵢ of one substrate depends on the concentration of the other substrate. The observed IC₅₀ values are therefore dependent on the concentrations of both substrates in the assay and their respective Kᵢ values. This will give large variations in the IC₅₀ values observed in different assays and makes direct comparison of inhibitors published in literature impossible. Correcting for the assay conditions by calculating the Kᵢ enables comparison between assays and assay conditions. The Kᵢ values were calculated from the IC₅₀ values using a model that takes into account that the enzyme operates via a ping-pong mechanism as observed in the enzyme kinetic study. It also takes into account that the inhibitors like AA affect only one form of the enzyme, EX, as shown by the kinetic model (Figure 5). According to Cheng and Prusoff, if these requirements are met, Equation 1 can be used, which includes the Kᵢ values of both substrates and their respective concentrations used in the assay (15). The Kᵢ values of the inhibitors were calculated (example calculation in SI) using Equation 1 in which Kᵢ is the Kᵢ of Ac-CoA at the concentration of histone substrate used in the assay (2.1 µM), Kᵢ is the Kᵢ of the histone substrate at the concentration of Ac-CoA used (71 µM) and A and B the concentrations of either substrate in the assay (4 and 60 µM respectively).

\[
IC_{50} = K_i \left(1 + \frac{K_a B}{K_b A} + \frac{A}{K_a}\right) 
\]

Eq. 1

The results show that the inhibitors 15 and 11d with the 10 carbon atom aliphatic tail bind slightly better than the inhibitors with the 15 carbon atom tail AA and 11e. The inhibitors with the 5 carbon atom tail 14 and 11c completely lose their affinity within the investigated range of concentrations as was observed in a previous study on KAT5 (26). This indicates that hydrophobic interactions with the aliphatic tail play a major role in the inhibitory potency of this type of compound for KAT8. The importance of hydrophobic interactions of the aliphatic tail is further confirmed by the loss of potency of compound 11c with the 5 carbon atom tail.
8b that includes an aliphatic alcohol in the aliphatic tail. The substitution position of the salicylate makes a small difference in the observed potencies with the best potency observed in the salicylate 6-position. We also note that replacing of the salicylate moiety by a 2-hydroxy acetophenone moiety (13) completely removed activity, stressing the importance of the salicylate functionality.

**Table 4:** Inhibitory potency of anacardic acid (AA) derivatives on KAT8 and p300. The inhibitory potency for KAT8 was measured using an assay based on fluorescent detection of CoA. The $K_i$ values were calculated an adaptation of the Cheng-Prusoff equation based on the postulated ping-pong mechanism and inhibitor kinetics (Equation 1). The inhibition of p300 was measured using an assay based on radiolabeling of the histone substrate. * The IC$_{50}$ of C646 is similar to the $K_i$ value reported in literature(34).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>KAT8 $K_i$ (µM)</th>
<th>p300 inhibition at 50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td><img src="structure.png" alt="Structure AA" /></td>
<td>64 ± 8.9</td>
<td>97 %</td>
</tr>
<tr>
<td>15</td>
<td><img src="structure.png" alt="Structure 15" /></td>
<td>37 ± 7.0</td>
<td>88 %</td>
</tr>
<tr>
<td>14</td>
<td><img src="structure.png" alt="Structure 14" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition up to the maximum tested dose of 200 µM</td>
</tr>
<tr>
<td>11e</td>
<td><img src="structure.png" alt="Structure 11e" /></td>
<td>79 ± 11</td>
<td>85 %</td>
</tr>
<tr>
<td>11d</td>
<td><img src="structure.png" alt="Structure 11d" /></td>
<td>57 ± 6.6</td>
<td>95 %</td>
</tr>
<tr>
<td>11c</td>
<td><img src="structure.png" alt="Structure 11c" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition up to the maximum tested dose of 200 µM</td>
</tr>
<tr>
<td>13</td>
<td><img src="structure.png" alt="Structure 13" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition up to the maximum tested dose of 200 µM</td>
</tr>
<tr>
<td>8b</td>
<td><img src="structure.png" alt="Structure 8b" /></td>
<td>157 ± 7.2</td>
<td>No inhibition at 50 µM</td>
</tr>
<tr>
<td>8a</td>
<td><img src="structure.png" alt="Structure 8a" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition at 50 µM</td>
</tr>
<tr>
<td>C646</td>
<td><img src="structure.png" alt="Structure C646" /></td>
<td>No inhibition at 400 µM</td>
<td>IC$_{50}$ = 0.32 µM*</td>
</tr>
</tbody>
</table>

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Inhibitors – p300

In order to assess the selectivity of the KAT8 inhibition by the compounds of this focused collection, we tested them for inhibitory potency on p300. Towards this aim we employed an assay based on radiolabeling of the histone substrate (Table 4). The reference compound C646 had an IC\(_{50}\) of 0.32 µM, which is consistent with literature (34). AA showed 97% inhibition at a concentration of 50 µM. The salicylic acid derivatives inhibited p300 as well and showed comparable SAR as observed for KAT8. This suggests a similar binding mode and interactions, although KAT8 and p300 are structurally very different. It is not possible to align either the amino acid sequence or the 3D structures of KAT8 and p300 by conventional means. It is however possible that either the Ac-CoA or histone substrate pockets, due to the similarity of the ligands, show a certain resemblance thus resulting in a comparable SAR for these inhibitors.

Conclusions

In this study, the catalytic mechanism of KAT8 histone acetyltransferase has been investigated. Enzyme kinetic experiments indicate that this bi-substrate enzyme operates by a ping-pong mechanism. This mechanism is supported by the observation that binding of the first substrate Ac-CoA is required for binding of the second substrate histone H4 as determined by ITC measurements. The presence of cysteine 143 in the KAT8 active site combined with the previous evidence that this residue is essential for catalysis further supports the evidence for ping-pong mechanism for acetyltransferase activity of KAT8. We employed this model for calculation of the Ki values of inhibitors of this enzyme. In order to generate small molecule inhibitors for KAT8 we assembled a focused compound collection around the known non-selective HAT inhibitor AA. This compound collection was tested for inhibition of KAT8. Kinetic studies were performed with the reference compound AA and based on both inhibitor kinetics and mechanistic studies, a catalytic model was proposed involving two different conformations of the enzyme. The equilibrium binding constant Ki was calculated using an adaptation of the Cheng-Prusoff equation based on the catalytic mechanism and the proposed model. AA and its derivatives inhibited KAT8 and both the aliphatic tail and the salicylate functionality proved to be important for binding. The inhibitors were tested for activity on p300 and showed a similar SAR as on KAT8, suggesting a similar binding mode even though the two enzymes are structurally different.

This study gives insight in KAT8 through the catalytic mechanism and presents a series of small-molecule inhibitors for this HAT. Based on kinetic studies of AA and the catalytic mechanism, a model has been proposed comprising the catalytic activity of KAT8 and the inhibitory action of AA, which includes an active and an inactive conformation of the enzyme. This provides a basis for development of inhibitors and the interpretation of the enzyme inhibition studies, which will ultimately enable the exploitation of KAT8 as a novel drug target in disease.
Acknowledgements

We thank Prof. Y. G. Zheng for kindly providing the KAT8 plasmid. We acknowledge the European Research Counsel for providing an ERC starting grant (309782) to F. J. Dekker. RF-2010-2318330 grant (A.M.), IIT-Sapienza Project (A.M.), FP7 Projects BLUEPRINT/282510 and A-PARADDISE/602080 (A.M.), Sapienza Ateneo Award Project 2014 (D.R.), PRIN 2012 (prot.2012CTAYSY) (D.R.).
Chapter 3

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Enzyme kinetics and inhibition of histone acetyltransferase KAT8


Chapter 3


Supporting Information

Biology

Expression and purification of KAT8-His

The KAT8 protein, carrying an N-terminal His-tag without linker sequence, was produced in E. Coli BL21(DE3) using the T7 expression system. BL21(DE3) was transformed with the expression plasmid pET19b (KAT8-His6) and grown overnight on ampicillin agar plate (50 µg/mL ampicillin) at 37 °C. A preculture of LB medium containing ampicillin (10 mL, 50 µg/mL ampicillin) was inoculated with 1 colony from the agar plate and grown overnight at 37 °C, 180 rpm. Fresh LB medium (1 L, 50 µg/mL ampicillin) was inoculated with the preculture and grown for 6 hours at 37 °C, 180 rpm. Expression of the KAT8-His6 gene was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 300 µM. The culture was incubated at 20 °C overnight, 180 rpm. The cells were harvested by centrifugation (20 minutes, 4000 rpm) and resuspended in lysis buffer (10 mM Tris pH 7.4, 750 mM NaCl, 1% glycerol, 1 mM 2-mercaptoethanol and Pierce EDTA-free protease inhibitor cocktail tablet). The cells were lysed by sonication for 2 times 30 seconds at 50% amplitude on a Branson digital sonifier W-250D and spun down 1 hour at 15000 x g. The supernatant was loaded on a gravity flow column containing 1 mL Ni-sepharose resin, calibrated with lysis buffer. The column was washed with 6 mL imidazole (50 mM) in lysis buffer and eluted with 2 mL imidazole (250 mM) in elution buffer (10 mM MES, 750 mM NaCl, 10 mM MgCitrate, 1 mM 2-Mercaptoethanol, 1 % glycerol pH 6.5). The protein was further purified by size-exclusion chromatography. The protein (1 mL ± 10 mg/mL) was loaded on a HiLoad 16/60 Superdex 200 pg (GE Healthcare) connected to a NGC Medium-Pressure Chromatography System (Bio-Rad) and eluted with elution buffer (elution peak at 78 mL). Purity was analyzed by SDS-PAGE (Figure S1, MW 40 kD), protein concentration was measured by UV280 or Pierce Coomassie Protein Assay (Thermo Scientific). Pure KAT8 was immediately aliquoted, flash-frozen in liquid nitrogen and stored at -80 °C. The protein was stable for at least 6 months.

Figure S1: Expression and purification of KAT8. 1 = lysate, 2 = pellet, 3 = Histrap wash, 4 = elution 1, 5 = elution 2, 6-10 = size-exclusion chromatography fractions, 11 = protein marker.
Isothermal titration calorimetry (ITC)

ITC experiments were done using 40 µM KAT8 in buffer (10 mM MES, 750 mM NaCl, 10 mM MgCitrate, 1 mM 2-Mercaptoethanol, 1% glycerol). As substrate, a peptide of amino acids 1-21 of the N-terminal histone H4 tail was used: SGRGKKGGKGLGKGGAKRHRK-NH2 (Pepscan, The Netherlands), referred to as histone H4 peptide. Acetyl coenzyme A sodium salt (Sigma-aldrich, USA) (Ac-CoA) was used as cofactor. The MicroCal iTC200 (Malvern) was equilibrated at 20 °C. Ac-CoA (400 µM) in the same buffer was titrated over KAT8 with 2 µL per injection, 20 injections in total, 0.5 µL pre-injection (Figure S2). A binding curve was generated using MicroCal ITC-ORIGIN Analysis Software by calculating the area under the peak (AUP) and baseline subtraction. Subsequently, histone H4 peptide (400 µM) dissolved in the same buffer was titrated over KAT8/Ac-CoA from the first experiment with 2 µL per injection, 20 injections in total, 0.5 µL pre-injection and a binding curve was generated. Then histone H4 peptide (400 µM) was titrated over KAT8 with 2 µL per injection, 20 injections in total, 0.5 µL pre-injection. A proper binding curve could not be generated. From the ITC data the change in enthalpy (ΔH) generated by the binding of the substrate was derived. Based on this, the change in entropy (ΔT*S) and the Gibb’s free energy (ΔG) were calculated using the equation ΔG = ΔH - ΔT*S (Figure S2).

Figure S2: ITC experiments. ITC injection peaks for Ac-CoA vs KAT8, histone H4 peptide vs KAT8/Ac-CoA and histone H4 peptide vs KAT8. Energy distribution for binding curves. The change in enthalpy (ΔH), the change in entropy (ΔS) and Gibb’s free energy (ΔG) were calculated form the ITC experiments. Binding of both substrates was due to an increase in entropy of the system.
**KAT8 activity assays**

Activity of KAT8 was measured using fluorescent chemical detection of coenzyme A (CoA). Acetyl coenzyme A sodium salt (Sigma-aldrich, USA) (Ac-CoA) was used as cofactor. As substrate, a peptide of amino acids 1-21 of the N-terminal histone H4 tail was used: SGRGGKGGKGLGKGGAKRHRK-NH₂ (Pepscan, The Netherlands), referred to as histone H4 peptide. 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM, Sigma-aldrich) was used to detect CoA produced in the enzymatic reaction(18). All dilutions were made in assay buffer (50 mM HEPES pH 7.4, 0.1 mM EDTA, 0.01 % TritonX-100). Substrates and, in case of inhibitor assays, inhibitors were added to a black 96-well plate (Costar flat-bottom black polystyrene). The enzyme reaction was initiated by adding KAT8 and incubated 15 minutes at room temperature. The reaction volume was 50 µL. The reaction was stopped by adding 2-propanol (50 µL). For detection of produced CoA, CPM was added (final concentration 12.5 µM in 0.4 % DMSO in assay buffer) and incubated 15 minutes at room temperature. The final volume was 200 µL. The fluorescence intensity was measured using a BioTek Synergy H1 hybrid plate reader. The excitation wavelength was 392 nm and the emission was measured at 470 nm, gain 50. Raw data were exported to Excel and analyzed in GraphPad Prism 5.0 software. All experiments were done in triplicate and repeated at least two times.

**Standard curve**

A standard curve was made using 2-mercaptoethanol (BME) as mimic for the product CoA (Figure S3). CPM (12.5 µM final) was added to a serial dilution of BME (0 - 12.5 µM final) with Ac-CoA (12.5 µM), recombinant KAT8 (25 nM) and 2-propanol (25%) in a total volume of 200 µL and incubated 15 minutes at room temperature. The fluorescence intensity was measured at an excitation wavelength of 392 nm and emission wavelength of 470 nm. The presence of Ac-CoA, KAT8 and 2-propanol did not influence the slope (data not shown). The baseline was subtracted, arbitrary fluorescence units (AFU) were plotted against the concentration of BME, a linear regression was done and the slope was determined, which was 2291.5 AFU/µM. The concentration was converted to pmol, giving 11.5 AFU/pmol of product. This factor was used to convert AFU to velocity in steady state kinetic experiments.

**Figure S3**: Standard curve using 2-mercaptoethanol (BME) as mimic for the product CoA.
**Km histone H4 peptide**

The Km of histone H4 peptide was determined using steady state kinetics. The AFU was determined at varying concentrations of histone H4 peptide (0 - 400 µM), KAT8 (570 nM) and Ac-CoA (4 µM). The reaction time was 15 minutes. The negative control contained no Histone H4 peptide, KAT8 (570 nM) and Ac-CoA (4 µM). The velocity was calculated using the conversion factor derived from the standard curve. The data were analyzed using GraphPad Prism. The negative control was subtracted from all data and the curve was fitted using non-linear regression, Michaelis-Menten. The Vmax was defined as the maximum velocity as extrapolated by the curve fit. The Km of histone H4 peptide was defined as the concentration of histone H4 peptide at which 50 % of maximum velocity was reached. The kcat was calculated using the following equation: 

\[ \text{Kcat} = \frac{\text{Vmax}}{\text{ET}} = \frac{20}{28.5} = 0.7 \text{ min}^{-1}. \]

**Catalytic mechanism**

The catalytic mechanism was determined using steady state kinetics. The Km of Ac-CoA and the Vmax of the enzyme were determined at varying concentrations of Ac-CoA (0 – 20 µM, two-times dilution series) with KAT8 (274 nM) and 15, 30, 60 and 90 µM histone H4 peptide respectively. Reaction time was 15 minutes and the reaction volume 50 µL. The negative control contained no Ac-CoA, KAT8 (274 nM) and 15, 30, 60 and 90 µM histone H4 peptide respectively. The data were analyzed using GraphPad Prism. The velocity was calculated using the conversion factor derived from the standard curve. The kcat was calculated using the number of enzyme molecules in pmol in the assay: 

\[ 274*1000*0.00005 = 13.7. \]

**Inhibitor kinetic studies**

The Km of Ac-CoA and Vmax of the enzyme were determined at varying concentrations of Ac-CoA (0 -20 µM, two-times dilution series) with KAT8 (264 nM), histone H4 peptide (60 µM) and 0, 80, 160 and 240 µM of AA (10% DMSO in buffer) respectively. The velocity was calculated using the conversion factor derived from the standard curve. The data were analyzed using GraphPad Prism. The negative control (0 µM Ac-CoA) was subtracted from all data and the curve was fitted using non-linear regression, Michaelis-Menten. Vmax was defined as the maximum velocity as extrapolated by the curve fit. The Km of Ac-CoA was defined as the concentration of Ac-CoA at which 50 % of maximum velocity was reached.

The concentration of histone H4 peptide that gives half-maximum velocity (K_{half}), the hill slope and the V_{max} of the enzyme were determined at varying concentrations of histone H4 peptide (0 - 200 µM, two-times dilution series) with KAT8 (274 nM), Ac-CoA (4 µM) and 0, 80, 160 and 240 µM of AA (10% DMSO in buffer) respectively. The velocity was
calculated using the conversion factor derived from the standard curve. The data were analyzed using GraphPad Prism. The negative control (0 µM histone H4 peptide) was subtracted from all data and the curve was fitted using non-linear regression, allosteric sigmoidal. The $V_{\text{max}}$ was defined as the maximum velocity as extrapolated by the curve fit. The Hill slope (H) is a measure for the steepness of the curve. The $K_{\text{half}}$ was calculated from the $K_{\text{prime}}$ as generated from the curve fit according to the equation $K_{\text{prime}} = K_{\text{half}}^H$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>log IC$_{50}$ (µM)</th>
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</thead>
<tbody>
<tr>
<td>AA</td>
<td><img src="image" alt="Structure of AA" /></td>
<td>2.33 ± 0.06</td>
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<td>2.09 ± 0.07</td>
</tr>
<tr>
<td>14</td>
<td><img src="image" alt="Structure of 14" /></td>
<td>&gt;2.8</td>
</tr>
<tr>
<td>11$^*$</td>
<td><img src="image" alt="Structure of 11$^*$" /></td>
<td>2.42 ± 0.06</td>
</tr>
<tr>
<td>11d</td>
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<td>2.28 ± 0.05</td>
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<tr>
<td>11c</td>
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<td>&gt;2.8</td>
</tr>
<tr>
<td>13</td>
<td><img src="image" alt="Structure of 13" /></td>
<td>&gt;2.8</td>
</tr>
<tr>
<td>8b</td>
<td><img src="image" alt="Structure of 8b" /></td>
<td>2.72 ± 0.02</td>
</tr>
<tr>
<td>8a</td>
<td><img src="image" alt="Structure of 8a" /></td>
<td>&gt;2.8</td>
</tr>
<tr>
<td>C646</td>
<td><img src="image" alt="Structure of C646" /></td>
<td>&gt;2.8</td>
</tr>
</tbody>
</table>

**Figure S4**: Single point screening and IC$_{50}$ curves of KAT8 inhibitor assay. Single point measurements were performed using a final concentration of 400 µM inhibitor in 10 % DMSO. IC50 measurements were performed using a two times serial dilution at a final concentration of 0 - 1000 µM inhibitor in 10 % DMSO in triplicate. Mean and standard deviations are plotted. *N = 2, **N = 3.
KAT 8 inhibitory potency assays

In all inhibitor assays, a final concentration of 4 µM Ac-CoA, 60 µM histone H4 peptide, 260 nM KAT8 was used. Single point measurements were performed using a final concentration of 400 µM inhibitor in 10 % DMSO. IC\textsubscript{50} measurements were performed using a two times serial dilution at a final concentration of 0 - 1000 µM inhibitor in 10 % DMSO. The positive control (100 % activity) contained Ac-CoA (4 µM), histone H4 peptide (60 µM), KAT8 (260 nM) and 10 % DMSO. The negative control (0 % activity), contained the same as the positive control, however KAT8 was boiled for 5 minutes at 90 °C to inactivate it and added after stopping the enzyme reaction. Raw data were analyzed using GraphPad Prism software. Negative controls were subtracted as baseline and data were normalized to the positive control. Concentrations were converted to log scale. The sigmoidal curves were fitted using non-linear regression, log [inhibitor] vs response (Figure S4). The IC\textsubscript{50} was defined as the concentration of inhibitor which gave 50 % inhibition (Table S1).

Calculation Ki

The K\textsubscript{i} values were calculated from the IC\textsubscript{50} values using equation 1. The IC\textsubscript{50} values were determined from the KAT8 inhibitor assays. The K\textsubscript{a} is the K\textsubscript{m} of Ac-CoA at 60 µM histone H4 peptide as calculated in the mechanism studies (2.1 µM), K\textsubscript{b} is the K\textsubscript{m} of histone H4 peptide at 4 µM Ac-CoA as derived from the steady state kinetic assay (71 µM). A is the concentration of Ac-CoA used in the assay (4 µM) and B the concentration of histone H4 peptide used in the assay (60 µM). For example AA showed a log IC\textsubscript{50} value of 2.33 µM. The IC\textsubscript{50} is 10\textsuperscript{2.33} = 214 µM. Solving the equation gives 64 µM (Figure S5). This was done for all inhibitors.

P300 assay

The anacardic acid derivatives were tested for inhibitory potency on the recombinant catalytic domain (aa 1284-1672) of human p300 (MW= 45.1 kDa) using radio-isotope-labeled \[^{3}H\]-Acetyl-CoA (PerkinElmer). The reaction buffer was 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 1% DMSO. The inhibitors (3% DMSO final) were added to a solution of recombinant catalytic domain of p300 (80 nM) and the histone H3 substrate (5 µM) using Acoustic Technology (Echo 550, LabCyte Inc. Sunnyvale, CA) in a 2-fold serial dilution starting at 200 µM. C-646 was tested in a 10-dose IC50 mode with 2-fold serial dilution starting at 25 µM. \[^{3}H\]-Acetyl-CoA (2.3 µM) was added to initiate the enzyme reaction (final volume 5 µL) and incubated 1 hour at 30 °C. The reaction mixture was applied onto filter paper (P-81, Upstate), washed with PBS to remove free \[^{3}H\]-Acetyl-CoA, dried and placed in vials containing scintillation fluid. Radioactive counts were recorded on Tri-Carb 2800TR Liquid Scintillation Analyzer (PerkinElmer) Raw data were exported to Excel and analyzed in GraphPad Prism.
Enzyme kinetics and inhibition of histone acetyltransferase KAT8

4.0 software. The percentage of residual enzyme activity at 50 µM of inhibitor was determined. For 13, 14 and 11c no inhibition was observed up to the maximum tested dose of 200 µM.

**Table S1**: Log IC$_{50}$ values of inhibitors on KAT8.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>KAT8 $K_i$ (µM)</th>
<th>p300 inhibition at 50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td><img src="image1" alt="Structure" /></td>
<td>64 ± 8.9</td>
<td>97 %</td>
</tr>
<tr>
<td>15</td>
<td><img src="image2" alt="Structure" /></td>
<td>37 ± 7.0</td>
<td>88 %</td>
</tr>
<tr>
<td>14</td>
<td><img src="image3" alt="Structure" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition up to the maximum tested dose of 200 µM</td>
</tr>
<tr>
<td>11e</td>
<td><img src="image4" alt="Structure" /></td>
<td>79 ± 11</td>
<td>85 %</td>
</tr>
<tr>
<td>11d</td>
<td><img src="image5" alt="Structure" /></td>
<td>57 ± 6.6</td>
<td>95 %</td>
</tr>
<tr>
<td>11c</td>
<td><img src="image6" alt="Structure" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition up to the maximum tested dose of 200 µM</td>
</tr>
<tr>
<td>13</td>
<td><img src="image7" alt="Structure" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition up to the maximum tested dose of 200 µM</td>
</tr>
<tr>
<td>8b</td>
<td><img src="image8" alt="Structure" /></td>
<td>157 ± 7.2</td>
<td>No inhibition at 50 µM</td>
</tr>
<tr>
<td>8a</td>
<td><img src="image9" alt="Structure" /></td>
<td>No inhibition at 400 µM</td>
<td>No inhibition at 50 µM</td>
</tr>
<tr>
<td>C646</td>
<td><img src="image10" alt="Structure" /></td>
<td>No inhibition at 400 µM</td>
<td>IC$_{50} = 0.32$ µM*</td>
</tr>
</tbody>
</table>
Figure S6: IC\textsubscript{50} curves of p300 inhibitor assay. All compounds were tested in 10-dose IC\textsubscript{50} mode with 2-fold serial dilution starting at 200 μM. The percentage of residual enzyme activity at 50 μM of inhibitor was determined; C-646 was tested in a 10-dose IC\textsubscript{50} mode with 2-fold serial dilution starting at 25 μM.

Chemistry

General

Chemicals were purchased from commercial suppliers and used without further purification. If required, glassware was oven-dried before use. Reactions in the microwave were carried out in a Biotage Initiator\textsuperscript{TM} Microwave Synthesizer. The reactions were monitored by thin layer chromatography (TLC) using Silica Gel 60 F254 aluminium sheets. TLC’s were visualised using UV light or KMnO\textsubscript{4} solution. Column chromatography was performed using MP Ecochrom Silica Gel 32-63, 60 Å. Products were analyzed by proton (\textsuperscript{1}H) and carbon (\textsuperscript{13}C) nuclear magnetic resonance (NMR), recorded on the Bruker Advance 500 MHz. Chemical shifts were reported as part per million (ppm) relative to residual solvent peaks (CDCl\textsubscript{3}, \textsuperscript{1}H δ = 7.26, \textsuperscript{13}C δ = 77.16; CD\textsubscript{3}OD, \textsuperscript{1}H δ = 3.31, \textsuperscript{13}C δ = 49.00).

Intermediate products were analyzed by electrospray ionization mass spectra (ESI-MS) using an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. Final products were analyzed by high resolution mass spectrometry (HRMS) on a LTQ-Orbitrap XL mass spectrometer with a resolution of 60,000 at m/z 400 at a scan rate of 1Hz.

Synthesis of anacardic acid derivatives

((pent-4-yn-1-yloxy)methyl)benzene (4a)

Sodium hydride (60% in mineral oil, 0.96 g, 24 mmol) was added to 4-pentyn-1-ol (1.1 mL, 12 mmol) in THF (5mL) at 0 °C under nitrogen atmosphere. The solution was stirred for several minutes and benzyl bromide (1.6 mL, 13 mmol) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 16 hours. The reaction was cooled to 0 °C and quenched with methanol followed by water untill all solid was dissolved. The mixture was extracted with ethyl acetate (2 x 50 mL) and washed with water and brine (2 x 50 mL). The combined organic layers were dried over
MgSO₄, filtered and concentrated under reduced pressure. The product was purified on silica gel (1 : 20 ethyl acetate : petroleum ether) and concentrated under reduced pressure to obtain 14 as colorless oil (1.95 g, 11 mmol). Yield: 92%. Rf = 0.49 (1 : 10 ethyl acetate : petroleum ether). ¹H NMR (500 MHz, CDCl₃) δ 7.41 – 7.27 (m, 5H), 7.33 – 7.26 (m, 1H), 4.53 (s, 2H), 3.59 (t, J = 6.2 Hz, 2H), 2.34 (td, J = 7.1, 2.6 Hz, 2H), 1.96 (t, J = 2.7 Hz, 1H), 1.85 (quint, J = 6.7 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 138.5, 128.9 (2x), 127.8, 127.6 (2x), 83.98, 73.01, 68.68, 68.51, 28.70, 15.33. For full characterization see(35).

5-(5-(benzyloxy)pent-1-yn-1-yl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (6a)

O
O

((pent-4-yn-1-yloxy)methyl)benzene (1.5 g, 8.8 mmol) and freshly distilled Et₂NH (1.6 mL, 16 mmol) were added to a solution of 2,2-dimethyl-5-trifluoromethanesulfonyl-2,4-dihydro-1,3-benzodioxin-4-one (2.6 g, 8 mmol), Cul (0.15 g, 0.8 mmol) and Pd(PPh₃)₂Cl₂ (0.3 g, 0.4 mmol) in anhydrous acetonitrile (4 mL) under argon atmosphere. The reaction mixture was stirred under microwave irradiation for 30 minutes at 100 °C, extracted with ethyl acetate (2 x 30 mL) and washed with brine (2 x 30 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated under reduced pressure. The product was purified by column chromatography (1 : 10 ethyl acetate : petroleum ether) and concentrated under reduced pressure to yield 15 as yellow oil (1.6 g, 4.6 mmol). Yield: 57%. Rf = 0.44 (1 : 4 ethyl acetate : petroleum ether). ¹H NMR (500 MHz, CDCl₃) δ 7.45 – 7.26 (m, 6H), 7.19 (d, J = 7.7 Hz, 1H), 6.90 (d, J = 8.3 Hz, 1H), 4.58 (s, 2H), 3.74 (t, J = 6.1 Hz, 2H), 2.68 (t, J = 7.0 Hz, 2H), 2.00 (m, 2H), 1.73 (s, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 159.0, 156.5, 138.7, 134.8, 128.9, 128.3 (2x), 127.7, 127.46, 126.2, 117.9, 116.6, 114.1, 105.5, 97.7, 79.0, 73.0, 68.9, 28.6, 25.7, 25.6, 16.9. MS (ESI): m/z 351 [M+H]+.

5-(5-hydroxypentyl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (7a)

O

Pd/C 10 wt. % (0.46 g, 4.6 mmol) was added to a solution of 5-(5-(benzyloxy)pentyl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (1.6 g, 4.6 mmol) in ethyl acetate (30 mL) under nitrogen atmosphere. The suspension was shaken for 24 hours at 40 °C under hydrogen atmosphere (3 bar) in a Parr apparatus. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The resulting oil was redissolved in DCM and washed with water. The product was purified by column chromatography (100% CH₂Cl₂...
and subsequently 100% ethyl acetate) yielding 16 as yellow oil (1.0 g, 3.7 mmol). Yield: 91%. R<sub>f</sub> = 0.25 (1 : 6 ethyl acetate : petroleum ether). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.42 (t, J = 7.9 Hz, 1H), 6.94 (d, J = 7.6 Hz, 1H), 6.83 (d, J = 8.2 Hz, 1H), 4.08 (t, J = 6.7 Hz, 2H), 3.17 – 3.04 (m, 2H), 2.06 (s, 2H), 1.73 – 1.66 (m, 8H), 1.55 – 1.37 (m, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 160.2, 157.1, 148.0, 135.1, 125.1, 115.2, 112.0, 105.0, 64.5, 34.2, 30.7, 28.4, 25.9, 25.6, 21.0. MS (ESI): m/z 265 [M+H]<sup>+</sup>.

2-hydroxy-6-(5-hydroxypentyl)benzoic acid (8a)

5N potassium hydroxide (1.8 mL, 8.5 mmol) was added to a solution of 5-(5-hydroxypentyl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (0.21 g, 0.85 mmol) in THF (10 mL). The reaction mixture was stirred for 6 days at 55 °C and acidified with 1N Aqueous HCl. The product was extracted with ethyl acetate (2 x 30 mL) and washed with water (2 x 30 mL). The combined organic layers were dried with MgSO<sub>4</sub>, filtered and concentrated under reduced pressure. The product was purified by column chromatography (100% ethyl acetate) and concentrated under reduced pressure to yield 17 as yellow solid (0.043 g, 0.20 mmol). Yield: 24%. R<sub>f</sub> = 0.26 (1 : 9 methanol : ethyl acetate). <sup>1</sup>H NMR (500 MHz, MeOD) δ 7.22 (t, J = 7.7 Hz, 1H), 6.71 (d, J = 7.7 Hz, 2H), 3.53 (m, 2H), 3.03 – 2.79 (m, 2H), 1.64 – 1.49 (m, 4H), 1.43 – 1.35 (m, 2H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 176.0, 169.6, 157.4, 141.7, 129.1, 118.0, 110.8, 58.0, 31.7, 28.5, 28.1, 22.1. Melting point: 88 °C. HRMS: m/z [M-H], calculated for C<sub>12</sub>H<sub>15</sub>O<sub>4</sub> 223.0975, found 223.0974.

Undec-10-ynoic acid (2)

Methyl-10-undecynoate (0.49 mL, 2.5 mmol) was added to a solution of lithium hydroxide (0.29 g, 12 mmol) in a 1 : 3 mixture of water : THF (25 mL). The yellow mixture was stirred at RT for 24 hours and then acidified with 1N aqueous HCl (20 mL). The product was extracted with ethyl acetate (2 x 20 mL) and washed with 1N aqueous HCl (2 x 20 mL) and water (20 mL). The combined organic layers were dried with MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to yield 19 as purple crystals (0.46 g, 2.5 mmol). Yield: 100%. R<sub>f</sub> = 0.25 (1 : 5 ethyl acetate : petroleum ether). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 2.34 (t, J = 6.8 Hz, 2H), 2.17 (td, J = 7.1, 2.6 Hz, 2H), 1.94 (t, J = 2.6 Hz, 1H), 1.65 – 1.61 (p, J = 7.4 Hz, 2H), 1.55 – 1.48 (quint, J = 7.4 Hz, 2H), 1.40 – 1.30 (m, 8H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 180.4, 84.6, 68.1, 34.1, 29.1, 28.9, 28.8, 28.6, 28.4, 24.6, 18.3.
Lithium aluminium hydride (0.19 g, 5.0 mmol) was added to dry THF (10 mL) at 0 °C under nitrogen atmosphere. Subsequently, undec-10-ynoic acid (0.46 g, 2.5 mmol) in dry THF (5 mL) was added dropwise over 20 minutes. The mixture was allowed to warm to room temperature, stirred for 2 hours and then quenched with ethyl acetate (5 mL) and water (5 mL). The mixture was filtered over a glass filter. The product was extracted with ethyl acetate (3 x 30 mL) and washed with and water (3 x 30 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated under reduced pressure to yield 20 as pink oil (0.40 g, 2.4 mmol). Yield: 97%. Rₑ = 0.24 (1 : 10 ethyl acetate : petroleum ether). ¹H NMR (500 MHz, CDCl₃) δ 3.60 – 3.57 (t, J = 7.1 Hz, 2H), 2.19 – 2.13 (td, J = 7.1, 2.6 Hz, 2H), 1.93 (t, J = 2.6 Hz, 1H), 1.56 – 1.47 (m, 4H), 1.32 (m, 10H).

[(undec-10-yn-1-yloxy)methyl]benzene (4b)

Sodium hydride 60% in mineral oil (0.19 g, 4.8 mmol) was added to a solution of undec-10-yn-1-ol (0.40 g, 2.4 mmol) in dry THF at 0 °C under nitrogen atmosphere. The mixture was stirred for 5 minutes and subsequently benzyl bromide (0.32 mL, 2.6 mmol) was added. The reaction was allowed to warm to room temperature and stirred for 18 hours. The reaction was quenched with methanol until all solids were dissolved, concentrated under reduced pressure and redissolved in ethyl acetate (20 mL). The product was extracted with ethyl acetate (2 x 25 mL) and washed with and water (3 x 25 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated under reduced pressure. The product was purified by column chromatography (1 : 12 ethyl acetate : petroleum ether and 100% ethyl acetate) and concentrated under reduced pressure to yield 21 as yellow crystals (0.29 g, 1.1 mmol). Yield: 46%. Rₑ = 0.93 (1 : 5 ethyl acetate : petroleum ether). ¹H NMR (500 MHz, CDCl₃) δ 7.40 (m, 5H), 4.56 (s, 2H), 3.52 (t, J = 6.6 Hz, 2H), 2.23 (td, J = 7.1, 2.6 Hz, 2H), 1.99 (t, J = 2.6, 1H), 1.68 (p, J = 7.4 Hz, 2H), 1.58 (p, J = 7.4 Hz, 2H), 1.44 (m, 4H), 1.35 (m, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 138.8, 129.1, 128.8, 128.4, 127.7, 127.5, 84.8, 72.9, 70.5, 68.2, 33.6, 29.8, 29.5, 29.1, 28.8, 28.5, 26.2, 18.4. MS (ESI): m/z 259 [M+H]+.
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5-(11-(benzyloxy)undec-1-yn-1-yl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (6b)

![Chemical Structure](image)

(11-(undec-10-yn-1-yloxy)methyl)benzene (0.29 mL, 1.1 mmol) and freshly distilled Et₂NH (0.21 mL, 2 mmol) were subsequently added to a solution of 2,2-dimethyl-5-trifluoromethanesulfonyl-2,4-dihydro-1,3-benzodioxin-4-one (0.33 g, 1.0 mmol), CuI (0.019 g; 0.1 mmol) and Pd(PPh₃)₂Cl₂ (0.07 g, 0.05 mmol) in anhydrous acetonitrile (1.5 mL) under argon atmosphere. The reaction mixture was stirred under microwave irradiation for 90 minutes at 100 °C, extracted with ethyl acetate (2 x 25 mL) and washed with water (2 x 25 mL) and brine (25 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated under reduced pressure. The product was purified by column chromatography (1:15 ethyl acetate : petroleum ether) and concentrated under reduced pressure which yielded with impurities as a yellow solid (0.21 g, 0.5 mmol).

The crude product was used in the next step without further purification. R_f = 0.21 (1:10 ethyl acetate : petroleum ether). Crude product (impurity): ¹H NMR (500 MHz, CDCl₃) δ 7.43 – 7.37 (m, 1H), 7.34 (m, 3H), 7.20 (d, J = 7.7 Hz, 1H), 6.86 (d, J = 8.2 Hz, 1H), 6.58 (d, J = 8.5 Hz, 1H), 6.32 (d, J = 8.0 Hz, 1H), 4.50 (s, 2H), 3.47 (t, J = 6.6 Hz, 2H), 3.33 - 3.35 (m, 4H), 2.55 – 2.47 (m, 2H), 1.72 (s, 6H), 1.70 (s, 6H), 1.16 (t, J = 7.1 Hz, 4H).

5-(11-hydroxyundecyl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (7b)

![Chemical Structure](image)

Pd/C 10 wt. % (0.1 g, 1 mmol) was added to a solution of 5-(11-(benzyloxy)undec-1-yn-1-yl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (0.2 g, 0.5 mmol) in ethyl acetate (30 mL) under nitrogen atmosphere. The suspension was shaken for 16 hours at 40 °C under hydrogen atmosphere (3 bar) in a PARR apparatus. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The product was purified by column chromatography (1:10 ethyl acetate : petroleum ether) to yield 23 as transparent oil and the hydrogenolysis was continued (0.12 g, 0.28 mmol). Yield: 60%. R_f = 0.54 (1:10 ethyl acetate : petroleum ether). ¹H NMR (500 MHz, CDCl₃) δ 7.41 (t, J = 7.6 Hz, 1H), 7.37 (m, 4H), 7.33 – 7.27 (m, 1H), 6.95 (d, J = 7.6 Hz, 1H), 6.82 (d, J = 8.1 Hz, 1H), 4.53 (s, 2H), 3.49 (t, J = 6.7 Hz, 2H), 3.16 – 3.08 (m, 2H), 1.72 (s, 6H), 1.64 (m, 4H), 1.43 – 1.29 (m, 14H).

Pd/C 10 wt. % (0.04 g, 0.4 mmol) was added to a solution of 5-(10-(benzyloxy)decyl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (0.1 g, 0.2 mmol) in ethyl acetate (25 mL) under nitrogen atmosphere. The suspension was shaken for 18 hours at 40 °C under hydrogen...
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atmosphere (3 bar) in a PARR apparatus. The reaction mixture was filtered over Celite and concentrated under reduced pressure to yield 24 as transparent oil (0.10 g, 0.28 mmol). Yield: 100%. \( R_f = 0.10 \) (1 : 5 ethyl acetate : petroleum ether). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 7.38 (t, \( J = 7.9 \) Hz, 1H), 6.92 (d, \( J = 7.7 \) Hz, 1H), 6.79 (d, \( J = 8.2 \) Hz, 1H), 3.62 (t, \( J = 6.7 \) Hz, 2H), 3.12 – 3.02 (m, 2H), 1.69 (s, 6H), 1.65 – 1.49 (m, 4H), 1.37 – 1.22 (m, 14H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 160.3, 157.1, 148.5, 135.1, 125.1, 115.1, 112.0, 104.9, 62.9, 34.4, 32.8, 31.2, 29.7, 29.6, 29.5 (2x), 29.4 (2x), 25.9, 25.7, 25.6. MS (ESI): \( m/z \) 349 [M+H]+.

2-hydroxy-6-(11-hydroxyundecyl) benzoic acid (8b)

Aqueous HCl was added (11M, 3 mL) to a solution of 5-(11-hydroxyundecyl)-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one (0.080 g, 0.26 mmol) in dioxane (20 mL). The reaction mixture was stirred for 20 hours at room temperature. The product was extracted with ethyl acetate (2 x 25 mL) and washed with 1N aqueous HCl (20 mL) and water (2 x 25 mL). The combined organic layers were dried with MgSO\(_4\), filtered and concentrated under reduced pressure. The product was purified by column chromatography (4 : 1 ethyl acetate : petroleum ether) and concentrated under reduced pressure to yield 25 as white crystals (0.040 g, 0.13 mmol). Yield: 46%. \( R_f = 0.71 \) (100% ethyl acetate). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 7.31 (t, \( J = 7.9 \) Hz, 1H), 6.84 (d, \( J = 8.2 \) Hz, 1H), 6.74 (d, \( J = 7.5 \) Hz, 1H), 3.71 (t, \( J = 6.5 \) Hz, 2H), 3.07 – 2.90 (m, 2H), 1.58 (m, 4H), 1.36 – 1.25 (m, 14H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 174.7, 163.3, 147.4, 134.7, 122.5, 115.6, 111.4, 63.2, 36.5, 32.2, 32.1, 29.7, 29.5, 29.0 (2x), 28.9, 28.8, 25.4. Melting point: 94 °C. HRMS: \( m/z \) [M-H], calculated for C\(_{18}\)H\(_{27}\)O\(_4\) 307.1914, found 307.1921.

Methyl 2-methoxy-6-(pent-1-yn-1-yl)benzoate (10c)

1-pentyne (0.27 g, 2.8 mmol) and freshly distilled Et\(_2\)NH (0.51 mL, 5.0 mmol) were added to a solution of methyl 2-iodo-6-methoxybenzoate (0.73 g, 2.5 mmol), CuI (0.05 g, 0.3 mmol) and Pd(PPh\(_3\))\(_2\)Cl\(_2\) (0.090 g, 0.13 mmol) in anhydrous acetonitrile (1.5 mL) under argon atmosphere. The reaction mixture was stirred under microwave irradiation for 30 minutes at 100 °C, extracted with ethyl acetate (2 x 20 mL) and washed with brine (2 x 25 mL). The combined organic layers were dried with MgSO\(_4\), filtered and concentrated under reduced pressure. The product was purified by column chromatography (1 : 8
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ethyl acetate : petroleum ether) and concentrated under reduced pressure to yield 26 as yellow oil (0.49 g, 2.1 mmol). Yield: 87%. $R_f = 0.35$ (1:5 ethyl acetate : petroleum ether).

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.84 (d, $J = 2.0$ Hz, 1H), 7.48 (dd, $J = 8.6, 2.1$ Hz, 1H), 6.89 (d, $J = 8.6$ Hz, 1H), 3.90 (s, 3H), 3.88 (s, 3H), 2.36 (t, $J = 7.0$ Hz, 2H), 1.66 – 1.58 (m, 2H), 1.04 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 166.0, 158.4, 136.4, 134.9, 120.1, 116.2, 112.0, 89.5, 79.4, 56.1, 52.0, 22.2, 21.3, 13.5. MS (ESI): $m/z$ 233.3 [M+H]$^+$, 482.2 [2M+NH$_4$]$^+$. 

**Methyl 2-methoxy-6-pentylbenzoate (11c first step)**

![Methyl 2-methoxy-6-pentylbenzoate](image)

Pd/C 10 wt. % (0.23 g, 2.3 mmol) was added to a solution of methyl 2-methoxy-6-(pent-1-yn-1-yl)benzoate (0.49 g, 2.1 mmol) in methanol (35 mL) under nitrogen atmosphere. The suspension was shaken for 16 hours at 40°C under hydrogen atmosphere (3 bar) in a PARR apparatus. The reaction mixture was filtered over Celite and concentrated under reduced pressure to yield 27 as yellow oil (0.50 g, 2.1 mmol). Yield: 97%. $R_f = 0.57$ (1:4 ethyl acetate : petroleum ether). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.62 (d, $J = 2.3$ Hz, 1H), 7.28 (dd, $J = 8.6, 2.3$ Hz, 1H), 6.92 (d, $J = 8.5$ Hz, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 2.66 – 2.52 (m, 2H), 1.68 – 1.58 (m, 2H), 1.35 (m, 4H), 0.92 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 166.9, 157.3, 134.7, 133.2, 131.3, 119.9, 112.2, 56.2, 51.8, 34.7, 31.4, 31.1, 22.5, 13.9. MS (ESI): $m/z$ 237.2 [M+H]$^+$, 490.6 [2M+NH$_4$]$^+$. 

**2-hydroxy-6-pentylbenzoic acid (11c final)**

![2-hydroxy-6-pentylbenzoic acid](image)

Boron tribromide 1M solution in DCM (10 mL, 10 mmol) was added dropwise to a solution of methyl 2-methoxy-6-pentylbenzoate (0.50 g, 2.1 mmol) in anhydrous acetonitrile at -78°C under nitrogen atmosphere. The reaction mixture was allowed to slowly warm to room temperature and stirred for 15 hours. The reaction was quenched with a few drops of water and sodium carbonate 1M in water (30 mL). The mixture was acidified with 1N aqueous HCl (20 mL), extracted with ethyl acetate (2 x 20 mL) and washed with water (2 x 20 mL) and brine (2 x 20 mL). The combined organic layers were dried over MgSO$_4$ and concentrated under reduced pressure. The product was purified by column chromatography (1:3 ethyl acetate : petroleum ether) and concentrated under reduced pressure to yield 28 as a white solid (0.23 g, 1.1 mmol). Yield: 53%. $R_f = 0.71$ (100% ethyl acetate). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.75 (s, 1H), 7.37 (d, $J = 8.4$ Hz, 1H), 6.97 (d, $J = 8.5$ Hz, 1H), 2.59 (t, $J = 7.7$ Hz, 2H), 1.70 – 1.56 (m, 2H), 1.37 (m, 4H), 0.94 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 175.3, 160.3, 137.4, 134.1, 130.0, 117.6, 111.0, 34.8, 31.3, 31.1, 22.5, 14.0. Melting point: 96°C. HRMS: $m/z$ [M-H], calculated for C$_{12}$H$_{15}$O$_3$.
1-decyne (0.50 mL, 2.8 mmol) and freshly distilled Et₂NH (0.51 mL, 5.0 mmol) were added to a solution of benzyl 2-(benzyloxy)-5-iodobenzoate (1.1 g, 2.5 mmol), CuI (0.053 g, 0.25 mmol) and Pd(PPh₃)₂Cl₂ (0.088 g, 0.13 mmol) in acetonitrile (1.5 mL) under argon atmosphere. The reaction was stirred under microwave irradiation for 30 minutes at 100 °C. The reaction mixture was extracted with ethyl acetate (2 x 20 mL) and washed with brine (3 x 20 mL). The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The product was purified by column chromatography (1 : 15 ethyl acetate : petroleum ether) to yield 7 as yellow oil (0.83 g, 1.8 mmol). Yield: 73%. Rf = 0.47 (5 : 1 petroleum ether : ethyl acetate). ¹H NMR (500 MHz, CDCl₃) δ 7.91 (d, J = 2.3 Hz, 1H), 7.48 – 7.29 (m, 11H), 6.95 (d, J = 8.6 Hz, 1H), 5.36 (s, 2H), 5.19 (s, 2H), 2.40 (t, J = 7.1 Hz, 2H), 1.67 – 1.54 (m, 2H), 1.45 (q, J = 7.5 Hz, 2H), 1.40 – 1.24 (m, 8H), 0.91 (t, J = 6.9 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 165.6, 157.4, 136.4, 136.3, 136.0, 135.1, 128.6 (2x), 128.5, 128.3 (2x), 128.1, 127.9 (2x), 127.1 (2x), 120.7, 116.5, 113.6, 89.9, 79.2, 70.7, 66.8, 31.9, 29.2, 29.1, 29.0, 28.8 (2x), 22.7, 19.4, 14.1. MS (ESI): m/z 455.3 [M+H]+, 472.3 [M+NH₄]+, 926.8 [2M+NH₄]+.

5-decyl-2-hydroxybenzoic acid (11d)

Pd/C 10 wt. % (0.2 g, 2 mmol) was added to a solution of benzyl 2-(benzyloxy)-5-(dec-1-yn-1-yl)benzoate (0.62 g, 1.8 mmol) in methanol (15 mL) and ethyl acetate (15 mL) under nitrogen atmosphere. The suspension was shaken for 19 hours at 40 °C under hydrogen atmosphere (3 bar) in a PARR apparatus. The reaction mixture was filtered over Celite and concentrated under reduced pressure and subsequently freeze-dried a total of 9h to obtain yield 10 as grey crystals (0.50 g, 1.8 mmol). Yield: 97%. Rf = 0.42 (100% ethyl acetate). ¹H NMR (500 MHz, CDCl₃) δ 7.73 (s, 1H), 7.37 (d, J = 7.7 Hz, 1H), 6.96 (d, J = 7.3 Hz, 1H), 5.28 (t, J = 7.2 Hz, 2H), 1.61 (m, 2H), 1.30 (m, J = 19.6 Hz, 14H), 0.90 (t, J = 6.7 Hz, 3H). ¹³C NMR (126 MHz, MeOD) δ 173.1, 157.0, 130.4, 129.9, 127.9, 116.4, 113.4, 32.7, 29.7, 29.6, 27.3 (2x), 27.3, 27.1, 26.9, 20.3, 11.1. Melting point: 95 °C. HRMS: m/z [M-H], calculated for C₁₇H₂₅O₃ 277.1809, found 277.1813.
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**Benzyl 2-(benzyloxy)-5-(pentadec-1-yn-1-yl)benzoate (10e)**

1-pentadecyn (0.72 mL, 2.8 mmol) and freshly distilled Et<sub>2</sub>NH (0.51 mL, 5.0 mmol) were subsequently added to a solution of benzyl 2-(benzyloxy)-5-iodobenzoate (1.1 g, 2.5 mmol), CuI (0.050 g, 0.25 mmol) and Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (0.090 g, 0.13 mmol), in anhydrous acetonitrile (1.5 mL) under argon atmosphere. The reaction was stirred under microwave irradiation for 30 minutes at 100 °C. The reaction mixture was extracted with ethyl acetate (2 x 15 mL) and washed with brine (2 x 30 mL). The combined organic layers were dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure. The product was purified by column chromatography (1 : 10 ethyl acetate : petroleum ether) to yield 6 as a white solid (1.2 g, 2.2 mmol). Yield: 88%. R<sub>f</sub> = 0.65 (5 : 1 petroleum ether : ethyl acetate). 1H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.91 (d, J = 2.2 Hz, 1H), 7.48 – 7.27 (m, 11H), 6.95 (d, J = 8.7 Hz, 1H), 5.37 (s, 2H), 2.40 (t, J = 7.1 Hz, 2H), 1.67 – 1.57 (m, 2H), 1.52 – 1.40 (m, 2H), 1.30 (m, 18H), 0.92 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, CDCl<sub>3</sub>) δ 165.5, 157.3, 136.3 (2x), 135.0 (2x), 128.5 (2x), 128.4, 128.2 (2x), 128.0, 127.8 (2x), 127.0 (2x), 120.9, 116.7, 113.8, 90.0, 79.3, 70.8, 66.79, 31.9, 29.7 (2x), 29.6 (3x), 29.5, 29.3, 29.2, 29.0 (2x), 28.8, 22.7, 19.4, 14.1. MS (ESI): m/z 525.6 [M+H]+, 542.6 [M+NH<sub>4</sub>]<sup>+</sup>, 1066.7 [2M+NH<sub>4</sub>]<sup>+</sup>.

**2-hydroxy-5-pentadecylbenzoic acid (11e)**

Pd/C 10 wt. % (0.2 g, 2 mmol) was added to a solution of benzyl 2-(benzyloxy)-5-(pentadec-1-yn-1-yl)benzoate (1.2 g, 2.2 mmol) in methanol (5 mL) and ethyl acetate (30 mL) under nitrogen atmosphere. The suspension was shaken for 16 hours at 40 °C under hydrogen atmosphere (3 bar) in a PARR apparatus. The reaction mixture was filtered over Celite and concentrated under reduced pressure to yield 9 as a white solid (0.62 g, 1.8 mmol). Yield: 80%. R<sub>f</sub> = 0.60 (1 : 1 petroleum ether : ethyl acetate). 1H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.74 (s, 1H), 7.37 (d, J = 8.2 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 2.59 (t, J = 7.7 Hz, 2H), 1.62 (m, J = 7.1 Hz, 2H), 1.36 – 1.28 (m, 24H), 0.91 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, CDCl<sub>3</sub>) δ 174.7, 160.4, 137.4, 134.1, 129.9, 117.6, 110.9, 34.9, 31.9, 31.4, 29.7 (2x), 29.7 (3x), 29.6, 29.5, 29.4, 29.1 (2x), 22.7, 14.1. Melting point: 90 °C. HRMS: m/z [M-H], calculated for C<sub>22</sub>H<sub>35</sub>O<sub>3</sub> 347.2591, found 347.2599.
2-(heptylthio)-1-(2-hydroxyphenyl)ethanone (13)

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1-heptanethiol (0.73 mL, 4.7 mmol) was added to a solution of potassium hydroxide (0.26 g, 4.7 mmol) in methanol under nitrogen atmosphere. The mixture was cooled to 0 °C and 2-bromo-2'-hydroxyacetophenone (1.0 g, 4.7 mmol) was added. White precipitate formed upon addition. The reaction mixture was allowed to warm to room temperature and stirred for 3 hours. Methanol was evaporated under reduced pressure. The product was redisolved in ethyl acetate (60 mL) and washed with water (3 x 60 mL). The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The product was purified on silica gel (1 : 20 ethyl acetate : petroleum ether) and concentrated under reduced pressure to yield 13 as colorless oil (1.1 g, 4 mmol). Yield: 85%. Rᶠ = 0.56 (1 : 8 ethyl acetate : petroleum ether). ¹H NMR (500 MHz, CDCl₃) δ 12.08 (s, 1H), 7.79 – 7.74 (dd, J = 8.1, J = 1.3, 1H), 7.55 – 7.48 (dt, J = 8.1, J = 1.3, 1H), 7.03 (d, J = 8.4 Hz, 1H), 6.97 – 6.90 (d, J = 8.4, 1H), 3.80 (s, 2H), 2.66 – 2.61 (t, J = 7.4, 2H), 1.65 – 1.60 (m, 2H), 1.39 (q, J = 7.4, 2H), 1.30 (m, J = 11.1, 5.0 Hz, 6H), 0.90 (t, J = 6.6 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 201.0, 163.2, 136.7, 130.5, 119.0, 118.8, 117.9, 37.0, 32.7, 31.7, 29.0, 28.8, 28.7, 22.6, 14.1. HRMS: m/z [M-H], calculated for C₁₅H₂₃O₃S 283.1362, found 283.1360.

References

