Engineering a Promiscuous Tautomerase into a More Efficient Aldolase for Self-Condensations of Linear Aliphatic Aldehydes

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The enzyme 4-oxalocrotonate tautomerase (4-OT) from Pseudomonas putida mt-2 takes part in a catabolic pathway for aromatic hydrocarbons, where it catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate into 2-oxohexa-3-enedioate. This tautomerase can also promiscuously catalyze carbon–carbon bond-forming reactions, including various types of aldol reactions, by using its amino-terminal proline as a key catalytic residue. Here, we used systematic mutagenesis to identify two hotspots in 4-OT (Met45 and Phe50) at which single mutations give marked improvements in aldolase activity for the self-condensation of propanal. Activity screening of a focused library in which these two hotspots were varied led to the discovery of a 4-OT variant (M45Y/F50V) with strongly enhanced aldolase activity in the self-condensation of linear aliphatic aldehydes, such as acetaldehyde, propanal, and butanal, to yield $\alpha,\beta$-unsaturated aldehydes. With both propanal and benzaldehyde, this double mutant, unlike the previously constructed single mutant F50A, mainly catalyzes the self-condensation of propanal rather than the cross-condensation of propanal and benzaldehyde, thus indicating that it indeed has altered substrate specificity. This variant could serve as a template to create new biocatalysts that lack dehydration activity and possess further enhanced aldolase activity, thus enabling the efficient enzymatic self-coupling of aliphatic aldehydes.

Introduction

The enzyme 4-oxalocrotonate tautomerase (4-OT) is composed of six identical subunits of only 62 amino acid residues each. It is a member of the tautomerase superfamily, a group of homologous proteins having a $\beta\alpha\beta$ structural fold and a catalytic amino-terminal proline (Pro1).4–5 4-OT takes part in a catabolic pathway for aromatic hydrocarbons in Pseudomonas putida mt-2, where it catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate (1, Scheme 1) into 2-oxohexa-3-enedioate (2).4–5 In this tautomerization reaction, Pro1 acts as a general base ($pK_a \approx 6.4$) in abstracting the 2-hydroxy proton of 1 and transferring it to the C5-position to give 2.4–5

Scheme 1. Proton-transfer reaction naturally catalyzed by 4-OT.

In addition to its natural tautomerase activity, 4-OT promiscuously catalyzes several carbon–carbon bond-forming reactions.4,9–12 These include various types of aldol reactions such as the self-condensation of propanal (3; Scheme 2), the cross-condensation of acetaldehyde (6) with benzaldehyde (12), the cross-coupling of propanal (3) and benzaldehyde (12), and the intramolecular cyclization of hexanedial (15) or heptanediol.10,12,16 For the self-condensation of 3, as well as the cross-condensation of 6 and 12, 4-OT catalyzes both the initial aldol coupling step to yield the $\beta$-hydroxaldehyde intermediate and the subsequent dehydration step to yield the final $\alpha,\beta$-unsaturated aldehyde.12,16 In the proposed mechanism for the aldolase activity of 4-OT, the active-site Pro1 residue functions as a nucleophile rather than as a base, and reacts with the carbonyl functionality of the aldehyde to form a covalent enamine intermediate.10,17 This intermediate reacts with another aldol in an inter- or intramolecular aldol addition, after which the final product (the aldol compound or the corresponding dehydrated compound) is released from the active site upon hydrolysis.

Enzyme promiscuity has great promise as a source of synthetically useful catalytic transformations,2,7 and can be exploited as a starting point to create new biocatalysts for cha-
lenging aldol reactions.\cite{18,19} Previously, we constructed several active-site mutants of 4-OT, including variant F50A, which had improved aldolase activity for the cross-condensation of 6 with 12.\cite{12,20} Here, we report the engineering of 4-OT into a more efficient aldolase for the self-condensation of linear aliphatic aldehydes such as acetaldehyde (6), propanal (3), and butanal (9; Scheme 2). For this, a large collection of single mutants of 4-OT was screened for mutations that give a strong improvement in the desired aldolase activity; then, identified “hotspot” positions were subjected to combinatorial mutagenesis.

**Results and Discussion**

We applied a systematic mutagenesis strategy to identify residue positions at which mutations give a marked improvement in the aldolase activity of 4-OT. For this, a collection of 1040 single mutants of 4-OT was used (at least 15 of the 19 possible variants at each residue position, from Ile2 to Arg62).\cite{21} Pro1 mutants were not included in the collection, because Pro1 is a key catalytic residue and mutations lead to incorrect demethionylation of the protein.\cite{22} The collection was screened for activity for the self-condensation of 3 under conditions that allow the detection of variants with strongly improved aldolase activity only (heatmap in Figure 1).

Two residue positions at which single mutations led to large improvements in aldolase activity were identified. The first was Met45: replacement with Thr, His, or Ile resulted in a pronounced improvement. The second was Phe50: substitution by Val strongly improved aldolase activity. In addition, replacement of His6 with Met and of Ala33 with Lys significantly improved aldolase activity. Mutant F50V showed the highest aldolase activity among all variants. Progress curves of the aldol self-condensation of 3 (50 mM) catalyzed by purified enzymes confirmed the enhanced activity of mutant F50V compared to wild-type 4-OT or the previously constructed mutant F50A (Figure 2).

In order to further improve the aldolase activity of 4-OT, we constructed a double-site library where the two hotspots, Met45 and Phe50, were simultaneously randomized by using NNK-codon degeneracy ($N = A/C/G/T$, $K = G/T$).\cite{23,24} to cover all 20 possible amino acids, for both positions. We also constructed a triple-site library in which His6, Met45, and Phe50 were randomized: NNK-codon degeneracy was used for His6 and Met45, whereas Phe50 was randomized by using NYK-codon degeneracy ($Y = C/T$).\cite{23,24} NYK degeneracy was chosen because it reduces the library size by covering only the codons of nine different aliphatic and polar amino acid residues, including those residues that result in enhanced aldolase activity, as well as the wild-type residue. The two libraries were transformed into *Escherichia coli* BL21(DE3) cells. Approximately 800 transformants from the double-site library and about 3500 from the triple-site library were screened for the aldol self-condensation of 3.

Screening of the double-site library identified M45Y/F50V with strongly enhanced aldolase activity (≈30-fold improvement over wild-type); the triple-site library did not yield a mutant with higher activity than that of M45Y/F50V. The progress curves of the aldol self-condensation of 3 (50 mM) confirmed the enhanced activity of M45Y/F50V compared to the single mutants F50V and F50A (Figure 2 and Figure S1 in the Supporting Information). $^1$H NMR spectroscopy confirmed the formation of 5 as the product of the aldol self-condensation of 3. Hence, 4-OT can be engineered into a more efficient aldolase for the self-condensation of 3 to yield 5 by exploring small libraries in which only hotspots are varied.

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**Scheme 2.** Examples of aldol reactions promiscuously catalyzed by 4-OT.
Next, we tested whether mutant M45Y/F50V also has enhanced aldolase activity towards the self-condensation of aldehydes 6 and 9. Wild-type 4-OT and mutants of 4-OT (P1A, F50A, and M45Y/F50V; 150 μm each) were incubated with either 6 or 9 (50 μm each), and the reactions were monitored by UV spectroscopy. The results indicate that the self-condensation of 6 or 9 (to give 8 or 11) is enzyme-catalyzed, with variant M45Y/F50V having the strongest activity (Figures 3 and 4). 

\[ \text{HNMR spectroscopy revealed the formation of 7 and 8 as products of the enzyme-catalyzed self-condensation of 6 (Figures S3 and S4), and the formation of 11 as a product of the enzyme-catalyzed self-condensation of 9 (Figures S5 and S6).} \]

Next, we compared the ability of mutants F50A and M45Y/F50V to catalyze an aldol cross-condensation reaction by using 3 and 12 as substrates. Mutants (150 μm) were incubated with 3 (50 μm) and 12 (2 mm), and the reactions were followed by UV spectroscopy. The UV spectra of the reaction mixture incubated with mutant F50A showed a decrease in absorbance at 250 nm (Figure 5A), thus indicating the depletion of 12 as the result of a cross-coupling reaction. Interestingly, the UV spectra for mutant M45Y/F50V showed a negligible decrease in absorbance at 250 nm (Figure 5B). Instead, an increase in absorbance at 234 nm was observed (corresponding to the formation of 5) as the result of the self-condensation of 3. 

\[ \text{1H NMR spectroscopic analysis confirmed that 13 and 14 were the main products in the reaction with F50A (indicative of cross-coupling), whereas 5 was the main product for M45Y/F50V (Figure S7).} \]

Hence, compared to the previously constructed mutant F50A,\[ ^{12} \text{mutant M45Y/F50V has altered substrate specificity and prefers the self-condensation of 3 over the cross-coupling of 3 and 12. This is fully consistent with the fact that mutant M45Y/F50V was engineered for enhanced activity towards the self-condensation of 3.} \]

In conclusion, we demonstrate that the promiscuous enzyme 4-OT can be engineered into a more efficient aldolase (variant M45Y/F50V) for self-condensations of aliphatic aldehydes by exploring small libraries in which only two hotspots (Met45 and Phe50) are varied. Notably, in our recent study, the
same positions were identified as hotspots for improving the aldolase activity of 4-OT for the cross-condensation of aldehydes 6 and 12: variant M45T/F50A was identified after mutagenesis and activity screening.\(^{20}\) Hence, 4-OT can be tailored to catalyze a specific aldol reaction. Work is in progress to determine the crystal structures of 4-OT variant M45Y/F50V in complex with 5, 8, or 11. Such enzyme–product structures could guide the design of variants that completely lack dehydration activity and possess further enhanced aldolase activity, thus enabling the efficient enzymatic self-coupling of small aliphatic aldehydes. With only a few natural aldolases available to catalyze the self-coupling of aldehydes, including 2-deoxyribose-5-phosphate aldolase (DERA)\(^{18b}\) and \(\alpha\)-fructose-6-phosphate aldolase (FSA),\(^{19b}\) the engineering of a promiscuous enzyme might prove to be a useful approach to create new biocatalysts for these challenging aldolizations.

Figure 3. UV spectra recorded after incubation of 6 with A) no enzyme (control), B) mutant P1A, C) wild-type 4-OT, D) mutant F50A, and E) mutant M45Y/F50V. The formation of 8 (\(\lambda_{\text{max}} = 227\) nm) was monitored.

Figure 4. UV spectra recorded after incubation of 9 with A) no enzyme (control), B) mutant P1A, C) wild-type 4-OT, D) mutant F50A, and E) mutant M45Y/F50V. The formation of 11 (\(\lambda_{\text{max}} = 240\) nm) was monitored.
Experimental Section

Materials: The sources for the buffers, solvents, components of lysozyme broth (LB) medium as well as the materials, enzymes and reagents exploited in molecular biology procedures are reported elsewhere.[23]

General methods: Standard molecular biology techniques were performed as previously described.[24] Protein in CFEs and purified samples was analyzed by PAGE using pre-casted NuPAGE 10% polyacrylamide gels (Novex; 10% Bis-Tris). Coomassie brilliant blue was used to stain the gels. Protein concentrations were determined by the Waddell method.[25] Enzymatic assays were performed in a V-650 or V-660 spectrophotometer (Jasco) or with a SPECTROstar Omega plate reader (BMG LABTECH). 1H NMR spectra were recorded on a Varian Inova 500 spectrometer (500 MHz) with a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in ppm and are referenced to H2O (δ = 4.80 ppm).

Expression and purification of wild-type 4-OT and 4-OT mutants: Wild-type 4-OT and 4-OT mutants were produced in E. coli BL21(DE3) as native proteins without an affinity tag, from either a pET20b(+) or pJexpress 414[21] expression vector. The construction of the expression vectors for wild-type 4-OT and 4-OT mutants P1A and F50A, as well as the purification procedure for 4-OT, were reported previously.[26,27]

Cell-free extract (CFE) preparation of E. coli cells expressing the single 4-OT mutants: A defined collection of 1040 single mutant 4-OT genes, each cloned into pJexpress 414, was purchased from BL21(DE3), as described elsewhere,[28] and stored at −80°C. Expression of the mutant 4-OT genes in E. coli BL21(DE3), the preparation of CFEs, and the assessment of concentrations of the mutants are described elsewhere.[21]

Determination of the aldolase activity: The aldolase activity in CFE was determined by UV spectroscopy in UV-star μclear 96-wells microtiter plates (MTP; Greiner Bio-one). The reaction mixtures consisted of CFE (30% v/v) and propanal (3, 40 mM) in NaH2PO4 buffer (100 μL, 10 mM, pH 7.3). MTPs were sealed with VIEWseal UV-transparent seals (Greiner Bio-one) to prevent evaporation, and incubated for 16 h at 25°C, with the reaction progress monitored by UV spectroscopy (220–500 nm). Formation of product 5 (λmax = 234 nm) was quantified based on the increase in absorbance at 234 nm. In order to eliminate false positives as a result of protein precipitation (in this case there is an increase in absorbance over the whole UV spectrum), reaction mixtures in which there was a high increase in absorbance at 350 nm (ΔA350 > 0.5) were assigned as “precipitation” (Figure 1). At this wavelength product 5 has no UV absorbance. The aldolase activity of wild-type 4-OT was used as a reference, and mutants with a marked improvement in aldolase activity were identified. The heat map (Figure 1) was prepared in Microsoft Excel 2010; the data are represented as colors for visual interpretation.

Construction of double- and triple-mutant libraries: The construction of the double and triple mutant libraries is described elsewhere.[20]

UV spectroscopic assay for the self-condensation of 3: The self-condensation of propanal (3) at RT was monitored for 20 h by following the increase in absorbance at 234 nm; this corresponds to the formation of 2-methylpent-2-enal (5). Enzyme (150 μM) was incubated in a 1 mm cuvette with 3 (50 mM) in NaH2PO4 buffer (0.3 mM, 20 mM, pH 7.3). UV spectra were recorded from 200–400 nm (Figures S1 and 2).

Redissolving WT 4-OT and 4-OT mutants in NaD2PO4 buffer: A ViVASPIN concentrate (5000 Da cut-off; Sartorius, Goettingen, Germany) was washed four times with H2O by centrifugation (1900g, 20 min). Subsequently, the concentrator was charged with enzyme (300 μL, –10 mg mL−1 in NaH2PO4 buffer (20 mM, pH 7.3)) and centrifuged (1900g, 30 min). The enzyme was retained on the filter, and then redissolved in NaD2PO4 (200 μL, 20 mM; pH 7.6) and centrifuged (1900g, 30 min). The remaining enzyme on the filter was redissolved in NaD2PO4 (300 μL, 20 mM; pH 7.6), and the final enzyme concentration was determined. Exchange of NaH2PO4 with NaD2PO4 was carried out only for enzyme preparations for the 1H NMR spectroscopic assay for the self-condensation of 3 or cross-coupling of 3 and 12.

1H NMR spectroscopic assay for the self-condensation of 3: Compound (290 μM) was incubated with 3 (30 mM) at RT in NaD2PO4 buffer (650 μL, 20 mM; pH 7.6) in an NMR tube, with [18]crown-6 ether as the internal standard (2.15 mM). A control sample was prepared containing all components except enzyme. 1H NMR spectra were recorded at about 1 h after the start of the incubation, and subsequently after 1, 4, 8, and 14 days.

Hydrated form of 3: 1H NMR (500 MHz, 20 mM NaD2PO4; pH 7.6): δ = 9.69 (t, J = 1.3 Hz, 1H), 2.57 (dq, J = 7.3, 1.3 Hz, 2H), 1.06 ppm (t, J = 7.3 Hz, 3H).

Internal standard [18]crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane): 1H NMR (500 MHz, 20 mM NaD2PO4; pH 7.6): δ = 3.68 ppm (2H, 4H).

UV spectroscopic assay for the self-condensation of 6: The self-condensation of 6 was monitored for 20 h at RT by following the increase in absorbance at 227 nm (formation of 8). Enzyme (150 μM) was incubated in a 1 mm cuvette with 6 (50 mM) in NaH2PO4 buffer (0.3 mL, 20 mM pH 7.3). UV spectra were recorded from 200–400 nm (Figure 3).

1H NMR spectroscopic assay for the self-condensation of 6: Compound (290 μM, in NaH2PO4 (20 mM)) was incubated with 6 (50 mM) at RT in NaD2PO4 buffer (650 μL, 20 mM; pH 7.6) in an NMR tube, with [18]crown-6 ether as the internal standard (2.15 mM). A control sample was prepared containing all components except enzyme. 1H NMR spectra were recorded approximate-
ly 2 h after the start of the incubation and then after 1, 4, and 7 days.

**Compound 6:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.66$ (q, $J = 3.0$ Hz, 1H), 2.23 ppm (d, $J = 3.0$ Hz, 3H).

**Hydrated form of 6:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 5.23$ (q, $J = 5.3$ Hz, 1H), 1.32 ppm (d, $J = 5.3$ Hz, 3H).

**Compound 7:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.69$ (t, $J = 5.9$ Hz, 1H), 5.16 (m, 1H), 2.03 (m, 2H), 1.18 ppm (d, $J = 5.9$ Hz, 3H).

**Compound 8:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.37$ (d, $J = 8.3$ Hz, 1H), 7.19 (m, 1H), 6.21 (m, 1H), 2.05 ppm (d, $J = 7.0$ Hz, 3H).

**UV spectroscopic assay for the self-condensation of 9:** The self-condensation of 9 was monitored for 20 h at RT by following the increase in absorbance at 240 nm (formation of 11). Enzyme (150 m$\mu$) was incubated in a 1 mm cuvette with 9 (50 m$\mu$) in Na$_2$PO$_4$ buffer (0.3 mM, 20 mM, pH 7.3). UV spectra were recorded from 200–400 nm (Figure 4A–E).

$^1$H NMR spectroscopic assay for the self-condensation of 9: Enzyme (290 m$\mu$ in Na$_2$PO$_4$ (20 mM)) was incubated with 9 (50 m$\mu$) at RT in Na$_2$PO$_4$ buffer (650 mM, 20 mM, pD 7.6) in an NMR tube, with [18]-crown-6 ether as the internal standard (2.15 mM). A control sample was prepared containing all components except enzyme. $^1$H NMR spectra were recorded approximately 2 h after the start of the incubation and then after 1, 4, and 7 days.

**Compound 9:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.65–9.67$ (m, 1H), 2.50 (m, 2H), 1.59–1.67 (m, 2H), 0.88–0.94 ppm (m, 3H).

**Hydrated form of 9:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 5.03$ (t, $J = 5.5$ Hz, 1H), 1.31–1.43 (m, 2H), 1.53–1.59 (m, 2H), 0.88–0.94 ppm (m, 3H).

**Compound 10:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.26$ (s, 1H), 6.75 (t, $J = 7.5$ Hz, 1H), 2.42 (q, $J = 7.3$ Hz, 2H), 2.25 (m, 2H), 1.55 (m, 2H), 0.90–0.97 (m, 3H), 0.90–0.97 ppm (m, 3H).

**UV spectroscopic assay for the cross-coupling of 3 and 12:** The cross-coupling of 3 and 12 was monitored for 5 h at RT by following the decrease in absorbance at 250 nm (depletion of 12) and the increase in absorbance at 234 nm (formation of 5). Enzyme (150 m$\mu$) was incubated in a 1 mm cuvette with 3 (50 m$\mu$) and 12 (2 m$\mu$) in Na$_2$PO$_4$ buffer (0.3 mL, 20 mM, pH 7.3). UV spectra were recorded from 200–400 nm (Figure 5).

$^1$H NMR spectroscopic assay for the cross-coupling of 3 and 12: Enzyme (290 m$\mu$) was incubated with 3 (30 m$\mu$) and 6 (15 m$\mu$) in Na$_2$PO$_4$ buffer (650 mL, 20 mM, pD 7.6) in an NMR tube, with [18]-crown-6 ether as internal standard (2.15 mM). A control sample was prepared with all the components except enzyme. $^1$H NMR spectra were recorded approximately 2 h after the start of the incubation, and then after 1, 4, 8, and 14 days.

**Compound 12:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.94$ (s, 1H), 7.97 (d, $J = 7.9$ Hz, 2H), 7.76 (d, $J = 7.5$ Hz, 1H), 7.63 ppm (dd, $J = 7.9, 7.5$ Hz, 2H).

**Compound 13:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.74$ (s, 1H), 7.48–7.36 (m, 5H), 5.23 (s, 1H), 1.03 ppm (s, 3H).

**Hydrated form of 13:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 7.48–7.36$ (m, 5H), 5.21 (s, 1H), 4.94 (s, 1H), 0.94 ppm (s, 3H).

**Compound 14:** $^1$H NMR (500 MHz, 20 mM Na$_2$PO$_4$; pD 7.6): $\delta = 9.49$ (s, 1H), 7.67 (d, $J = 7.5$ Hz, 2H), 7.55–7.50 (m, 4H), 2.03 ppm (s, 3H).

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**Conflict of Interest**

The authors declare no conflict of interest.

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[18] a) T. D. Machajewski, C.-H. Wong, Angew. Chem. Int. Ed. 2000, 39, 1352 – 1375; Angew. Chem. 2000, 112, 1406 – 1430; b) C. F. Barbas III, Y.-F. Wang, C.-H. Wong, J. Am. Chem. Soc. 1990, 112, 2013 – 2014.

[19] a) P. Clapés, W.-D. Fessner, G. A. Sprenger, A. K. Samland, Curr. Opin. Chem. Biol. 2010, 14, 154 – 167; b) X. Garrabou, J. A. Castillo, C. Guérard-Hélaine, T. Parella, J. Joglar, M. Lemaire, P. Clapés, Angew. Chem. Int. Ed. 2009, 48, 5521 – 5525; Angew. Chem. 2009, 121, 5629 – 5633.

[20] M. Rahimi, J.-Y. van der Meer, E. M. Geertsema, H. Poddar, B.-J. Baas, G. J. Poelarends, ChemBioChem 2016, 17, 1225 – 1228.

[21] J.-Y. van der Meer, H. Poddar, B.-J. Baas, Y. Miao, M. Rahimi, A. Kunzendorf, R. van Merkerk, P. G. Tepper, E. M. Geertsema, A.-M. W. H. Thunnissen, W. J. Quax, G. J. Poelarends, Nat. Commun. 2016, 7, 10911.

[22] B.-J. Baas, E. Zandvoort, A. A. Wasiel, G. J. Poelarends, FEBS Open Bio 2014, 4, 651 – 658.

[23] M. T. Reetz, M. Bocola, J. D. Carballeira, D. Zha, A. Vogel, Angew. Chem. Int. Ed. 2005, 44, 4192 – 4196; Angew. Chem. 2005, 117, 4264 – 4268.

[24] M. T. Reetz, D. Kahakeaw, R. Lohner, ChemBioChem 2008, 9, 1797 – 1804.

[25] H. Raj, B. Weiner, V. Puthan Veetil, C. R. Reis, W. J. Quax, D. B. Janssen, B. L. Feringa, G. J. Poelarends, ChemBioChem 2009, 10, 2236 – 2245.

[26] J. Sambrook, E. F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press 1989.

[27] W. J. Waddell, J. Lab. Clin. Med. 1956, 48, 311 – 314.

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