

# Characterization of the enzyme activity and substrate specificity of GLYAT haplotypes

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## **PREFACE**

To my heavenly Father, my God I would like to give all the credit to You. I thank You for this opportunity and for the grace You have given me.

I would like to express my gratitude and thanks for my study leader Dr Rencia van der Sluis. Thank you for the last couple of years where you have taught me so much, given me guidance and helping me.

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## ABSTRACT

The unavoidable exposure to xenobiotics makes detoxification a necessity. Detoxification is not just one reaction but four reactions (Phase 0, I, II and III) that work together. The glycine conjugation pathway is one of the Phase II detoxification pathways. The glycine conjugation pathway plays an important role in detoxification xenobiotic substances such as benzoate and salicylate. Benzoate can be found in foods and drinks in the form of additives and preservatives and salicylate is found in aspirin. This pathway is responsible for making the substances less lipophilic for ease of excretion in the urine. The glycine conjugation pathway is made up of two different enzymes, the medium chain fatty acid: CoA ligase (ASCM2B, E.C. 6.2.1.2) enzyme and the glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13) enzyme. This pathway is theorised to be critical for survival as there are only 14 haplotypes, indicating that the pathway is highly conserved, and no defects in this pathway have been reported. This study focused on the second enzyme GLYAT, which is responsible for the conjugation of substances with glycine.

GLYAT has the highest substrate affinity for benzoyl-CoA compared to other substances such as salicyl-CoA, isovaleryl-CoA, propionyl-CoA and butyryl-CoA. Previous studies found that single nucleotide polymorphisms (SNPs) can effect enzyme activity. The S<sub>156</sub> variant is the suggested wild-type variant with the highest relative enzyme activity and the highest allele frequency. The S<sub>17T</sub> variant had the third highest relative enzyme activity and the R<sub>199C</sub> variant was barely active. The aim of this study was to evaluate whether the conserved haplotypes (S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub>), that were identified in the worldwide genetic variation analyses, have similar effects on the enzyme activity as the SNPs that were characterised. The kinetic mechanism of GLYAT in previous studies used one substrate at saturating concentration while varying the other substrate. In this study both substrates, benzoyl-CoA and glycine, were varied using a wide range of concentrations.

This S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub> variants were already available in the laboratory. The T<sub>17</sub>S<sub>156</sub> variant was constructed using site directed mutagenesis with the S<sub>156</sub> variant template. All three proteins were successfully expressed in Origami\_pGro7 cells and purified using N-terminal Trx-His-tags. The purification was needed as purified proteins are needed for enzyme kinetic reactions. Relative enzyme activity for all three proteins were determined, S<sub>156</sub> had the highest relative enzyme activity when compared to the other two variants (4.19μM/min). T<sub>17</sub>S<sub>156</sub> had the second highest activity (2.6μM/min) followed by S<sub>156</sub>C<sub>199</sub> with the lowest activity (1.7μM/min). The bi-substrate kinetics was determined with varying substrate concentrations after a global fit of data was performed, the sigmoidal enzyme kinetic model had the best fit. The substrate specificity was calculated and S<sub>156</sub> had the highest substrate specificity for glycine of 19.89 s<sup>-1</sup>mM<sup>-1</sup> and for benzoyl-CoA of 4.46 s<sup>-1</sup>

$^1\text{mM}^{-1}$ , T<sub>17</sub>S<sub>156</sub> had the second highest substrate specificity for both glycine and benzoyl-CoA with  $11.01 \text{ s}^{-1}\text{mM}^{-1}$  and  $2.68 \text{ s}^{-1}\text{mM}^{-1}$  respectively. S<sub>156</sub>C<sub>199</sub> had the lowest substrate specificity values with  $1.65 \text{ s}^{-1}\text{mM}^{-1}$  for glycine and  $0.66 \text{ s}^{-1}\text{mM}^{-1}$  for benzoyl-CoA. The kinetic studies can help to classify GLYAT with regards to how the different haplotypes will have an influence on the enzyme activity as well as the substrate specificity in order to help understand the function of the enzyme *in vivo*.

Keywords: Glycine N-acyltransferase; detoxification; glycine conjugation pathway, haplotypes, relative enzyme activity; bi-substrate kinetics.

## OPSOMMING

Die onvermydelike blootstelling aan xenobiotika maak detoksifisering noodsaaklik. Detoksifisering is nie net een reaksie nie, maar vier reaksies wat saamwerk; die glysienkonjugasieweg is een van die Fase II-prosesse in die detoksifiseringsweg. Die glysienkonjugasieweg speel 'n belangrike rol in die detoksifisering van xenobiotiese stowwe soos benzoaat en salisilaat. Benzoaat word gevind in voedsel en drankies in die vorm van byvoegmiddels en preserveermiddels, en salisilaat word in aspirien gevind. Hierdie weg is verantwoordelik vir die vermindering van lipofieliese stowwe sodat dit gemaklik in die uriene uitgeskei kan word. Die glysienkonjugasieweg bestaan uit twee verskillende ensieme, die mediumketting-vetsuur: CoA-ligase (ASCM2B, E.C. 6.2.1.2) ensiem en die glysienasieltransferase (GLIAT, E.C. 2.3.1.13) ensiem. Dit word voorspel dat hierdie weg van kritieke belang is vir oorlewing, want daar is slegs 14 haplotipes, wat daarop dui dat die weg baie bewaard is, en dat daar geen afwyking in hierdie weg aangemeld is nie. Hierdie studie fokus op die tweede ensiem GLIAT, wat verantwoordelik is vir die konjugasie van stowwe met glysien.

GLIAT het die hoogste affiniteit vir bensoïel-KoA in vergelyking met ander stowwe soos salisiel-KoA, isovaleriel-KoA, propioniel-KoA en buturiel-KoA. Vorige studies het bevind dat enkelnukleotied-polimorfismes (ENPs) ensiemaktiwiteit kan beïnvloed. Die S<sub>156</sub> variant is die voorgestelde wildtipe variant met die hoogste relatiewe ensiemaktiwiteit en die hoogste alleelfrekwensie. Die S<sub>17</sub>T variant het die derde hoogste relatiewe ensiemaktiwiteit gehad en die R<sub>199</sub>C variant was skaars aktief. Die doel van hierdie studie was om te evalueer of die gekonserveerde haplotipes (S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> en S<sub>156</sub>C<sub>199</sub>), wat geïdentifiseer is in die wêreldwye analise van genetiese variasie, soortgelyke effek op die ensiemaktiwiteit sal hê soos die ENPs wat gekenmerk is. Die kinetiese meganisme van GLIAT het een substraat in versadigde konsentrasie gebruik terwyl die ander substraat konsentrasie verander is. In hierdie studie sal beide substrate, bensoïel-KoA en glysien, met 'n wye verskeidenheid konsentrasies gevarieer word.

Die S<sub>156</sub> en S<sub>156</sub>C<sub>199</sub> variante was reeds in die laboratorium beskikbaar. Die T<sub>17</sub>S<sub>156</sub> variant is gekonstrueer deur gebruik te maak van polimerase ketting reaksies (PKR) met die S<sub>156</sub> variant as templaar. Al drie proteïene is suksesvol in Origami\_pGro7 selle uitgedruk en gesuiwer met behulp van N-terminale Trx-His-tags. Die suiwing was nodig, aangesien gesuiwerde proteïene nodig is vir ensiemkinetiese reaksies. Relatiewe ensiemaktiwiteit vir al drie proteïene is bepaal, S<sub>156</sub> het die hoogste relatiewe ensiemaktiwiteit gehad as dit vergelyk word met die ander twee variante (4,19 μM). T<sub>17</sub>S<sub>156</sub> het die tweede hoogste aktiwiteit gehad (2,6 μM), gevolg deur S<sub>156</sub>C<sub>199</sub> met die laagste aktiwiteit (1,7 μM). Die tweesubstraat-kinetika is met verskillende substraatkonsentrasies bepaal nadat 'n wêreldwye passing van data uitgevoer is, en die sigmoïedale ensiemkinetiese model het die beste gepas. Die substraatspesifisiteit is bereken en die resultate dui aan dat S<sub>156</sub>

die hoogste substraatspesifisiteit het vir glisien met 'n waarde van  $19,89 \text{ s}^{-1}\text{mM}^{-1}$  en vir bensoïel-KoA 'n waarde van  $4,46 \text{ s}^{-1}\text{mM}^{-1}$  gehad, T<sub>17</sub>S<sub>156</sub> het die tweede hoogste substraatspesifisiteit gehad vir beide glisien en bensoïel-KoA met waardes van  $11,01 \text{ s}^{-1}\text{mM}^{-1}$  en  $2,68 \text{ s}^{-1}\text{mM}^{-1}$  onderskeidelik. S<sub>156</sub>C<sub>199</sub> het die laagste substraat-spesifisiteitwaardes met 'n waarde van  $1,65 \text{ s}^{-1}\text{mM}^{-1}$  vir glisien en  $0,66 \text{ s}^{-1}\text{mM}^{-1}$  vir bensoïel-KoA.

Die kinetiese studies kan help om GLIAT te klassifiseer ten opsigte van hoe die verskillende haplotipes 'n invloed op die ensiemaktiwiteit en die substraatspesifisiteit sal hê om die funksie van die ensiem *in vivo* te help verstaan.

Sleutelwoorde: Glisienasieltransferase; detoksifisering; glysienkonjugasieweg, haplotipes, relatiewe ensiemaktiwiteit; tweesubstraat-kinetika.

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## CHAPTER 1 INTRODUCTION

In modern day most people are constantly exposed to xenobiotic. These xenobiotic are exogenous and endogenous, and they need to be detoxified to be excreted, if not a toxic build-up can occur in the body. It is important that detoxification takes place, which is a multi-step process in the body (Liska *et al.*, 2006). Every pathway that is part of detoxification plays a vital role, every gene that is responsible for enzymes in this pathway as-well.

The glycine conjugation pathway plays an important role in the human body. This pathway is mainly responsible for the conjugation of benzoate and salicylate to form more excretable end-products (Nandi *et al.*, 1979; Mawal & Qureshi, 1994; Schachter & Taggart, 1953). The enzymes in the pathway is ACSM2B and GLYAT, ACSM2 is responsible for producing the acyl-CoA and GLYAT will conjugate the acyl-CoA with glycine (Schachter & Taggart, 1953; Knights, 1998; Vessey *et al.*, 1999; Knights & Drogemuller, 2000).

Previous studies done on GLYAT was done on unknown variants of the enzyme or on recombinantly expressed enzymes containing only one single nucleotide polymorphism (SNP's) (Kelley & Vessey, 1994; Mawal & Qureshi, 1994; van der Westhuizen *et al.*, 2000; Matsuo *et al.*, 2012; van der Sluis *et al.*, 2013). This study was done to determine how three of the different haplotypes of GLYAT (S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub>.) will influence the workings of GLYAT, and whether these haplotypes increase or decrease the relative enzyme activity. What the role of the haplotypes are on the enzyme kinetic model. These are all factors that will help us understand how to characterise GLYAT.

### Chapter 2:

This chapter is the literature review. This chapter explains detoxification of xenobiotics that need to be excreted to ensure that no toxic build-up can occur. The glycine conjugation pathway is part of this detoxification process, it forms part of Phase II detoxification. The glycine conjugation pathway is mainly responsible for the detoxification of benzoyl-CoA and glycine and is a two-step process. ACSM2B is the first step and GLYAT the second step. A detailed discussion of both of these enzymes are given and how they work. The problem statement followed by the aim and objective is also given in this chapter.

### **Chapter 3:**

In this chapter, the experimental procedure, results and discussion for the construction, expression and purification of  $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$  are described.

### **Chapter 4:**

The experimental procedures, results and discussion for the relative enzyme kinetics, bi-substrate kinetics and kinetic mechanism for  $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$  with substrates benzoyl-CoA and glycine.

### **Chapter 5:**

Conclusion and future prospects.

### **References:**

The references used throughout the dissertation in the Harvard format.

### **Appendix:**

Added graphs of the bi-substrate kinetics with the raw data.

### **Aims**

To characterise the kinetic parameters and substrate specificity of  $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$  haplotypes identified in GLYAT.

### **Objectives**

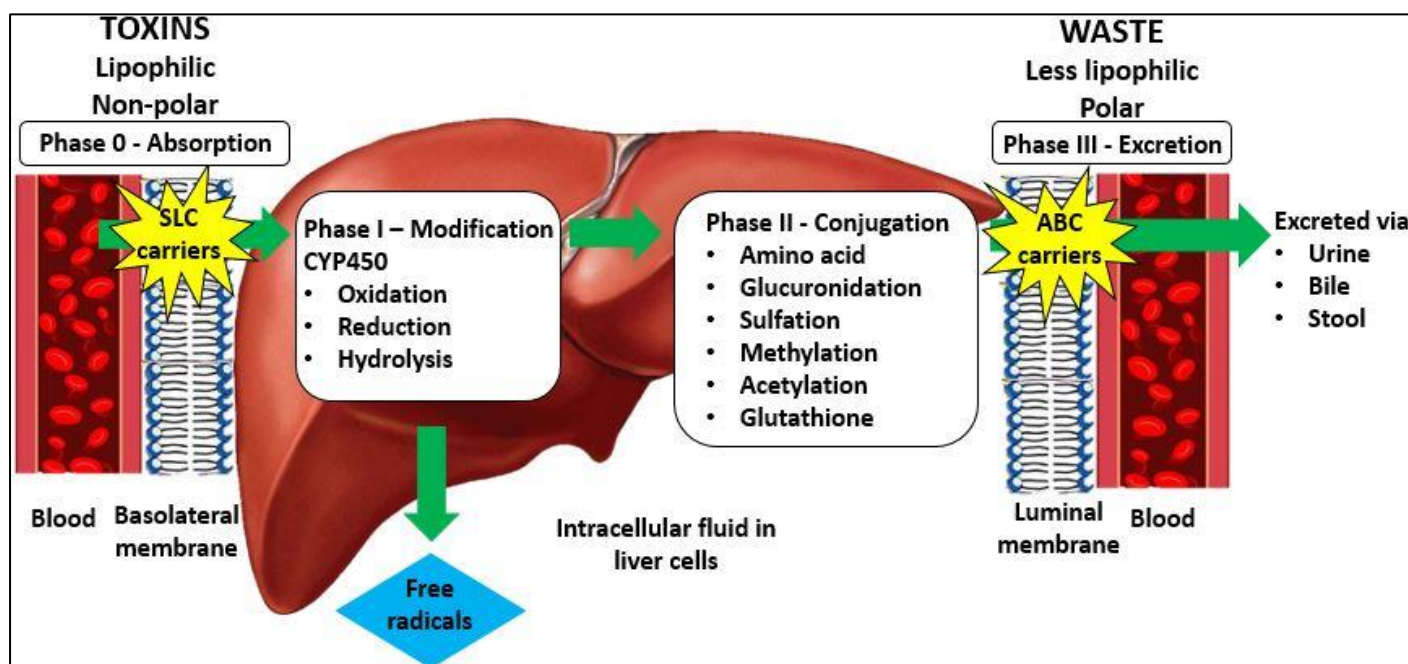
1. Construct, express and purify  $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$  recombinant GLYAT variants in bacteria.
2. Determine whether or not the haplotypes ( $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$ ) have an effect on the enzyme activity of GLYAT by analysing the relative activities.
3. Determine the bi-substrate (glycine and benzoyl-CoA) kinetics for  $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$
4. Determine the substrate specificity of the recombinant variants using benzoyl-CoA

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Biotransformation reactions

Constant and unavoidable exposure of exogenous and endogenous xenobiotics is the reason detoxification is required. The more lipophilic substances makes it difficult to be transported across the membranes of cells leading to non-efficient. The pressure of detoxification of these compounds have increased enormously. Detoxification is not just one reaction, but rather a process that involves multiple reactions and multiple players. The detoxification process will change toxic compounds by making them less lipophilic and more polar so that they can be easily excreted by the body. The process is referred to as biotransformation and is used for a vast number of xenobiotics. These include endotoxins that is the intermediate or end product of normal metabolism and gut bacteria, and exotoxins that are ingested, inhaled and absorbed toxins such as drugs, alcohol, food additives, pesticides, micro-organisms etc. (Reid & Evans, 2012).

In 1947 Roger Williams defined the detoxification field after putting together many observations. He described how there are two phases in detoxification, functionalization and conjugation which later became known as phase I and II (Liska *et al.*, 2006). It was later discovered that the uptake and elimination plays an important role in detoxification and two extra phases were added, phase 0 and III. Xenobiotic elimination requires the combined processes of metabolism and transport and is divided into four phases (Phase 0, I, II and III) (Doring and Petzinger, 2014) (Figure 2.1)



**Figure 2.1:** Schematic representation of the four different phases of detoxification of endogenous and exogenous chemicals such as food additives, drugs, alcohol, insecticides and micro-organisms, pesticides

and metabolic end products. Phase 0 is uptake into the liver, Phase I is modification, Phase II is conjugation and Phase III is excretion. Adapted from Badenhorst *et al.* (2014)

## 2.2 Phase 0

Phase 0 is the first phase and is known as the absorbance phase. When the drugs enter the blood system and are transported to the liver this phase is responsible for absorbing the compounds at the blood-facing basolateral membrane and is histologically separated from the bile-facing canalicular membrane and the urine-facing tubule brush border membrane. Phase 0 transporters are mainly of the solute carrier SLC21/SLCO organic anion transporting family – OATPs, with 52 SLC families in mammals currently known. The drugs are then transferred to a metabolising cell where Phase I reactions can occur. Phase 0 is known as “secondary active” meaning the drugs are transported down the concentration gradient of the transported substrate (Doring & Petzinger, 2014).

## 2.3 Phase I

Cytochrome P450 (CYP450) enzymes are involved in this phase and consists of a family of enzymes located mainly in the endoplasmic reticulum and mitochondria of most cells, mainly the liver. This enzyme family consists of approximately 50-100 different enzymes with the primary function of modifying endogenous and exogenous chemicals for excretion. The CYP450 family of enzymes are generally the first line of defence against foreign compounds. The synthesis of cytochrome is dependent on the amount of toxins that are digested by the body on a daily basis. These enzymes are responsible for changing lipophilic toxins into intermediates that will be more water-soluble (Liska *et al.*, 2006).

Phase I reactions will consist of oxidation, reduction or hydrolysis depending on the molecular structure of the compound. These reactions will lead to the exposure or addition of a functional group resulting in the compound that is more polar. This means that a water-soluble reactive site will be exposed for phase II conjugation. A significant side effect of phase I detoxification is the production of free radicals as the toxins are transformed. For each molecule of toxin metabolized by phase I, one free radical molecule is generated (Reid & Evans, 2012).

The intermediate phase, between phase I and II, is where the compounds are usually rendered more toxic and dangerous than before and would need to move quickly to phase II in order to be conjugated. These intermediate phase compounds can interact with DNA and proteins and can be harmful to biological systems. If phase I has a high activity, before the molecules are presented to phase II, there will be an increase in free radical production. Phase I activity should not be too high as this will influence phase II and can cause depletion of the glutathione conjugation, the

activity should also not be too slow as this can cause secondary tissue damage (Reid & Evans, 2012).

## 2.4 Phase II

Phase II is made up of several conjugation reactions including, glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. Phase II conjugation pathways consist of the cells adding another substance (such as cysteine, glycine or a sulphur molecule) to a toxic chemical or drug, to render it less harmful and ready to be excreted (Hagen *et al.*, 1990). Any phase II reaction will require high ATP levels. Phase I will react to form a reactive site and phase II is responsible for adding a water-soluble group to this reactive site.

Glucuronidation is the most common metabolic process for drugs and other xenobiotics, while sulphation is an important synthetic reaction, making some compounds more soluble by binding the substance. Glutathione conjugation or mercapturic acid formation is an enzymatic process resulting from the conjugation of reactive intermediates with glutathione, the conjugated product is then converted into mercapturic acid and excreted (Liska *et al.*, 2006). Glycination's (amino acid conjugation) responsibility is to facilitate the biotransformation of for example salicylates and benzoic acids (Reid & Evans, 2012).

The enzymes that are mainly used in phase II detoxification are transferases including UDP - glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acyltransferases (NATs), glutathione S-transferase (GSTs) and some methyltransferases (Jancova *et al.*, 2010).

### 2.4.1 Amino acid conjugation

The conjugation with amino acids is highly based on the animal species as well as the structure of the carboxylic acid of the xenobiotic. The highest occurrence of amino acid conjugates are the conjugates with glycine. In the light of this glycine is utilized by most animal species as well as a wide range of carboxylic acids i.e. aliphatic, aromatic, heteroaromatic and phenylacetic acid derivatives (J.Hutt & Caldwell, 1990).

Ornithine conjugation differs from the other amino acid conjugation reactions with both amino groups undergoing acylation. Taurine, not strictly an amino acid, also forms peptide bonds with xenobiotic acids (J.Hutt & Caldwell, 1990). Glutamine conjugation in mammals appears to be confined to arylacetic acids like phenylacetic acid and related compounds. An exception to this is diphenylmethoxyacetic acid, which yields a glutamine conjugate in the Rhesus monkey (Drach & Howell, 1968; Drach *et al.*, 1970).

## 2.5 Phase III

This is seen as the excretion phase. The compounds in this phase are already water soluble and need to be excreted. ATP binding cassette (ABC) carriers are the backbone for this phase and will perform the final step of drug excretion into fluids such as stool, urine and bile. The ABC transporters are comprised of seven families with around 20 carriers that are also involved in the transport of drugs. These carriers generate an active transport process giving phase III the “active” transport term. The transport process is uphill against the concentration gradient of the transported substance; this is possible by the expense of ATP. The conjugated metabolites are excreted via a transporter pump like MRP2, multidrug resistance-associated protein 1(MRP1)/P-gp and BCRP (Doring & Petzinger, 2014).

## 2.6 Glycine conjugation pathway

The two main compounds that the glycine conjugation pathway needs to eliminate are benzoic acid and salicylate (Nandi *et al.*, 1979; Mawal & Qureshi, 1994). Benzoic acid is usually ingested as sodium benzoate in the form of food additives, alcohol and drugs (Jay, 2000). Benzoic acid will form hippurate, a compound easily excreted (Schachter & Taggart, 1953). Salicylate will also be conjugated with glycine via this pathway, but not to the same extent as benzoic acid (Levy, 1965). Salicylate is a component in aspirin. Once aspirin is ingested it is hydrolysed to salicylic acid in the gastrointestinal tract (Ouellette & Joyce, 2010). Salicylic acid will be conjugated with glycine to form salicyluric acid (Amsel & Levy, 1969).

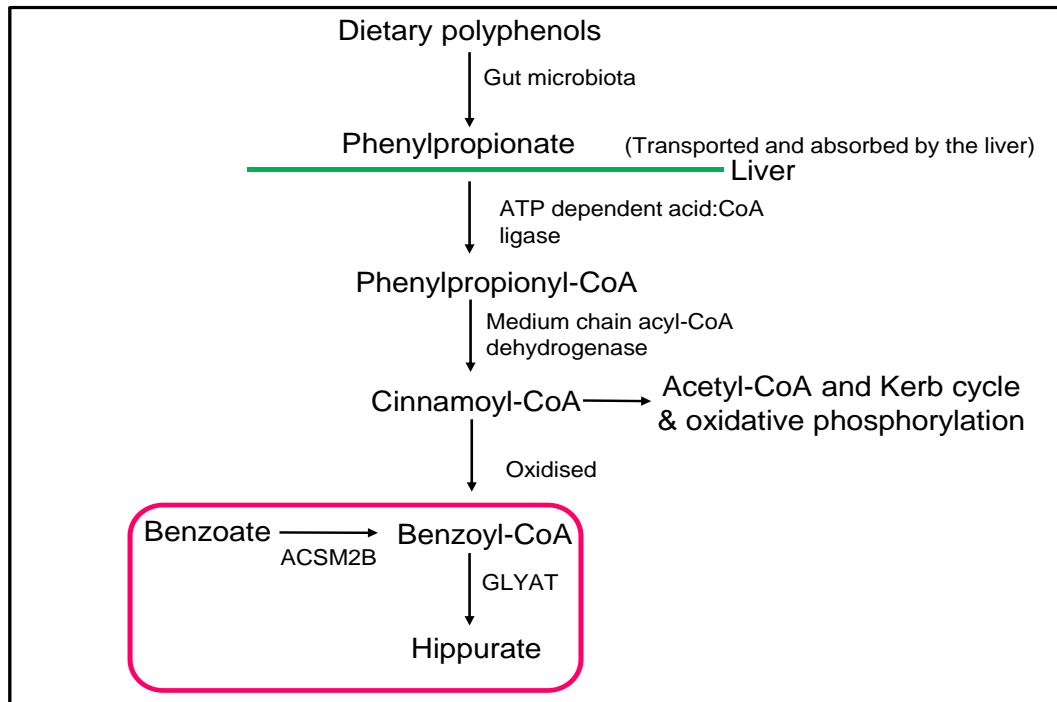
### 2.6.1 The history of the glycine conjugation pathway

In 1829 Liebig discovered hippuric acid in the urine of horses (reviewed in Conti & Bickel, 1977). Alexander Ure in 1841 discovered that when benzoic acid was ingested it resulted in the excretion of hippurate in the urine (Ure, 1841). In 1842 William Keller took 32 grains of pure benzoic acid syrup and could isolate hippuric acid the next morning in his urine confirming the findings made by Ure (Keller, 1842). In 1845 Dessaignes successfully established the structure of hippuric acid by boiling it with inorganic acids that resulted in the components being split into benzoic acid and glycine. Later Liebig discovered that hippuric acid is a normal urinary product found in humans consuming a mixed diet (reviewed in Conti & Bickel, 1977).

### 2.6.2 Phenylpropionate and glycine conjugation

Dietary polyphenols are converted by the gut microbia to simple aromatic acids like phenylpropionate. Phenylpropionate is then transported to and absorbed by the liver and metabolised to phenylpropionyl-CoA by the ATP dependent acid:CoA ligases. These acyl-CoA

trioesters are converted to cinnamoyl-CoA which in turn will be oxidised to benzoyl-CoA. Cinnamoyl-CoA is then used again in the Krebs cycle as well as the beta-oxidation pathway. Conjugation of benzoyl-CoA with glycine will form hippurate as the end product (Badenhorst *et al.*, 2014). Figure 2.2 shows the dietary polyphenols and how this pathway and the daily intake of benzoate are incorporated together.



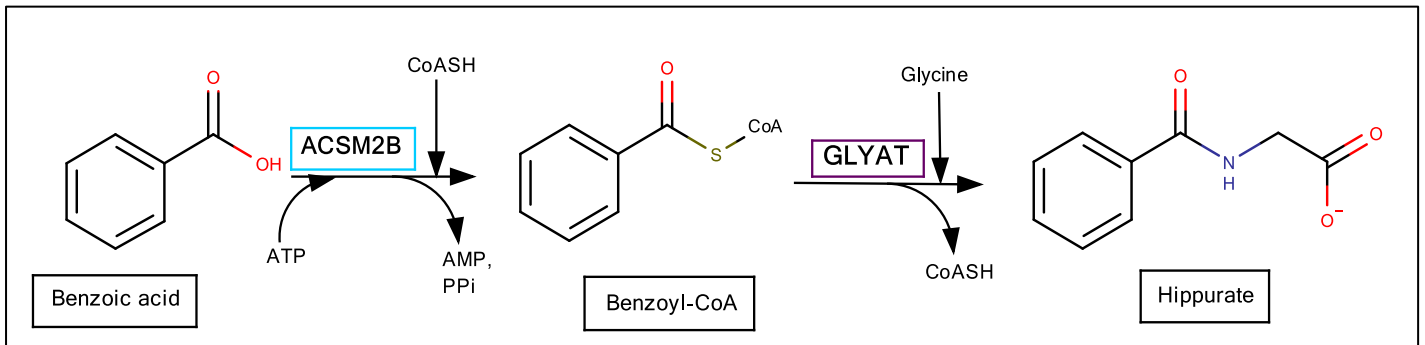
**Figure 2.2: Phenylpropionate and glycine conjugation.** The pathway of dietary polyphenols that is transformed into hippurate. The figure also indicates how the two pathways intercept. The glycine conjugation pathway is indicated in the pink box. Adapted from Badenhorst *et al.* (2014).

The pink box shows glycine conjugation of benzoate that is ingested on a daily basis. The recommended daily intake for benzoate is 5 mg per kg of body weight, but in populations that consumes high concentrations of preserved foods, benzoate can be as high as 280%. These findings together with the dietary polyphenols also ending in benzoyl-CoA can cause the glycine conjugation pathway to be under stress (Chiple, 2005; Lees *et al.*, 2013).

### 2.6.3 Reaction of the glycine conjugating pathway

The entire process of conjugating benzoic acid to form hippurate is a two-step reaction. This conjugating reaction will take place in the mitochondria of liver cells. According to Lees *et al.* (2013) the most abundant amino acid conjugate excreted in the urine of mammals is hippurate (Lees *et al.*, 2013). Firstly, benzoate will be activated to benzoyl-CoA by the medium chain fatty acid: CoA ligase (ACSM2B) enzyme. This enzyme is a mitochondrial ATP dependent acid:CoA ligase (E.C. 6.2.1.2) (Schachter & Taggart, 1953; Knights, 1998; Vessey *et al.*, 1999; Knights & Drogemuller,

2000). Secondly, the glycine N-acyltransferase enzyme (GLYAT, E.C. 2.3.1.13) will bind to benzoyl-CoA. This will catalyse the acylation of glycine to form an end product of hippurate



(Schachter & Taggart, 1953; Schachter & Taggart, 1954b; Schachter & Taggart, 1954a). In Figure 2.3 the reaction with all the components can be seen.

**Figure 2.3:** Basic reaction of the glycine conjugation pathway. Benzoic acid is converted to benzoyl-CoA by ACSM2B with the use of ATP. Benzoyl-CoA is then conjugated with glycine by GLYAT to form hippurate. Adapted from Badenhorst *et al.* (2013).

#### 2.6.4 Factors influencing the glycine conjugation pathway

The three main factors that can influence the rate and capacity of the pathway is ATP, CoASH and glycine. The first reaction, activation of benzoate to benzoyl-CoA, needs an ample amount of available CoASH. The availability of CoASH can directly limit the formation of hippurate excretion. The formation of benzoyl-CoA also requires ATP and is thus dependent on the availability of ATP. The ATP molecules are hydrolysed to AMP leading to two molecules of ATP consumed for one molecule of benzoate (Schachter & Taggart, 1953; Schachter & Taggart, 1954b). Nandi suggested that the activation of the acyl-CoA is more than likely the rate-limiting step in amino acid conjugation but also suggested that the transfer step might become the rate-limiting step in some genetic disorders (Nandi *et al.*, 1979).

The use of glycine is important in the formation of hippurate. Inadequate levels of glycine can directly limit the formation of hippurate while the administration of glycine can increase the rate of hippurate formation (Knights & Minors, 2012). Schachter and Taggart noted that in the absence of glycine there was no demonstrated formation of hippurate (Schachter & Taggart, 1953). Griffith discovered that when rats were fed sodium benzoate the rats with a glycine insufficient diet either died, did not illustrate sufficient growth and the formation of hippurate decreased. When glycine was added to the diet, the toxicity of benzoic acid declined and an increase in hippurate synthesis was seen (Griffith, 1929). All the above factors, glycine, ATP and CoASH, are necessary and crucial for successful glycine conjugation to proceed.

Variation in the two genes *ACSM2B* and *GLYAT* also has an influence on the rate of glycine conjugation. Any genetic variation that might occur in these genes, along with variation in the expression levels and enzyme activity of both *ACSM2B* and *GLYAT* can contribute to inter-individual variation in glycine conjugation (Badenhorst *et al.*, 2014).

## 2.7 Acyl-CoA synthetase

The first reaction in the glycine conjugation pathway, is the formation of an acyl Coenzyme A thioester that is mediated by an acyl-CoA synthetase (ACS) or ATP-dependent acid: CoA ligase with the main function to ligate a fatty acid to a CoA. These ligases are split into three ATP-dependent systems with the ACS as standard following the letter of the specific chain length of the carbon. First is the short chain or acetyl-CoA synthetase or acetate: CoA ligase (ACSS EC 6.2.1.1) of C<sub>2</sub>-C<sub>4</sub> in length. Secondly, the medium chain or butyryl-CoA synthetase or medium-chain fatty acid: CoA ligase (ACSM EC 6.2.1.2) C<sub>4</sub>-C<sub>12</sub> and optimal activity at C<sub>7</sub>. The long chain fatty acids of C<sub>12</sub>-C<sub>20</sub> are activated by long-chain fatty acyl-CoA synthetase or acyl CoA synthetase, or long chain fatty acid: CoA ligase (ACSL EC 6.2.1.3), and one GTP dependent, medium long chain fatty acid: CoA ligase (GDP) (EC 6.2.1.10) system (J.Hutt & Caldwell, 1990; Knights & Minors, 2012). ACSM showed activity for aromatic carboxylic acids (benzoic and phenylacetic acids) and several branched chain aliphatic acids. The ACSM gene family consist of 7 units, ACSM 1 – 6 (Schachter & Taggart, 1954b).

### 2.7.1 ACSM2

ACSM2 can be divided into ACSM2A and ACSM2B. The *ACSM2A* and *ACSM2B* genes encode nearly identical proteins with an amino acid identity of 97,6%. The nucleotide sequences are 98,8% alike. They are both found on chromosome 16p12.3 but *ACSM2A* is located on the forward strand and *ACSM2B* on the reverse strand (Watkins *et al.*, 2007). The *ACSM2B* gene is made up of more than 40 000 base pairs and contains 15 exons of which 13 are coding exons.

The ACSM2B enzyme is encoded by the *ACSM2B* gene. ACSM2B is the first enzyme in the glycine conjugation pathway. The function of this enzyme is to catabolise the reaction of benzoic acid and CoASH to form benzoyl-CoA (Knights, 1998; Knights & Drogemuller, 2000). This reaction requires high levels of ATP as two ATP molecules are consumed for one benzoate molecule (Schachter & Taggart, 1953; Schachter & Taggart, 1954b).

## 2.8 GLYAT

Schachter and Taggart (1954) first discovered glycine N-acyltransferase (GLYAT, EC 2.3.1.13) by partially purifying the enzyme from beef liver mitochondria (Schachter & Taggart, 1954a). GLYAT was purified from a human liver mitochondria preparation in 1970 (Tishler & Goldman, 1970).

Webster *et al.* (1976) isolated and purified two acyl-CoA: amino acid N-acyltransferases from Rhesus monkey and human liver mitochondrial fractions (Webster *et al.*, 1976). GLYAT is the second enzyme in the glycine conjugation pathway that is responsible for the conjugation of benzoyl-CoA with glycine (Schachter & Taggart, 1953; Schachter & Taggart, 1954b; Schachter & Taggart, 1954a).

### 2.8.1 Genetics of GLYAT

The *GLYAT* gene is located on chromosome 11q12 on the reverse strand and consist of 23 200 base pairs containing 6 exons and encodes the GLYAT enzyme. There are 2064 single nucleotide polymorphisms (SNPs) of which 145 are non-synonymous SNPs and 43 are synonymous (www.ensembl.org, September 2019, ENST00000344743.8). Human GLYAT has two splice variants coding *isoform a* with 296 amino acids and *isoform b* (does not contain exon 6) with 163 amino acids. No evidence has been found to prove isoform b is a functional protein (Lino Cardenas *et al.*, 2010).

There are similar proteins to GLYAT, with GLYAT having two homologous genes, GLYAT-like 1 (GLYAT-L1) and GLYAT-like 2 (GLYAT-L2) both on chromosome 11q12.1. GLYAT-L1 was discovered to code for the glutamine-conjugation enzyme with phenylacetyl-CoA glutamine N-acyltransferase activity (Lino Cardenas *et al.*, 2010; Matsuo *et al.*, 2012).

The Open Reading Frame (ORF) for *GLYAT* is encoded by exon 2 to 6. It is known that the GLYAT ORF is highly conserved with only 14 haplotypes identified to date (van der Sluis *et al.*, 2015). The haplotypes with the highest allele frequencies are S<sub>156</sub> (70%), T<sub>17</sub>S<sub>156</sub> (20%), wild-type (5%) and H<sub>131</sub>S<sub>156</sub> (0.9%). The haplotype frequency for S<sub>156</sub>C<sub>199</sub> is 0.05%. The phylogenetic analyses of GLYAT also suggested that the S<sub>156</sub> haplotype is the ancestral allele (van der Sluis *et al.*, 2015). Lino *et al.* (2010) suggested that the S<sub>156</sub> haplotype should be considered the new wild-type, according to van der Sluis *et al.* (2015) this haplotype has the highest enzyme activity as well as haplotype frequency across all of the population groups supporting Lino's suggestion that S<sub>156</sub> is the wild-type (Lino Cardenas *et al.*, 2010; van der Sluis *et al.*, 2015).

### 2.8.2 Organic acidemias

Organic acidemias are a metabolic disorder and can be classified as acyl-CoA's that accumulate to toxic levels. This is the cause of an enzyme being absent or the enzyme malfunctioning. Tanaka *et al.* (1966) first discovered isovaleric academia with the enzyme isovalery-CoA dehydrogenase being faulty. In 1967, they discovered that isovalerylglycine is excreted in patients with isovaleryl academia (Tanaka & Isselbacher, 1967). Isovaleryl-CoA is one of the preferred substrates for GLYAT, since isovaleryl-CoA builds up in the body the isovaleryl-CoA will conjugate with glycine

to form isovalerylglycine that will then be excreted (Schachter & Taggart, 1954a; Tanaka & Isselbacher, 1967). In 1971 Rasmussen and colleagues found propionic acidemias to be excreted as propionylglycine that has not been found previously in human bodily fluids (Rasmussen *et al.*, 1972). Propionic-CoA Carboxylase is the defective enzyme, leading to the formation of propionic-CoA build-up and propionylglycine (Gompertz *et al.*, 1970; Hsia *et al.*, 1970; Ando *et al.*, 1971).

Bartlett and Gompertz (1974a) observed that GLYAT does not only conjugate benzoic acid and salicylate, but other acyl-CoAs which is associated with organic acidemias. This led to their investigation of the specificity of GLYAT towards these acyl-CoAs (Bartlett & Gompertz, 1974a). It was discovered that both isovaleryl-CoA and propionyl-CoA are substrates for GLYAT. Isovaleryl-CoA will easily be converted to isovalerylglycine but propionyl-CoA was converted to propionylglycine at very slow rates and little is excreted (Tanaka *et al.*, 1966; Bartlett & Gompertz, 1974b; Krieger & Tanaka, 1976; Fenton *et al.*, 2001).

### 2.8.3 GLYAT expression in hepatocellular carcinoma

A study showed that the expression of GLYAT was reduced in hepatocellular carcinomas. This indicated that GLYAT can be related to hepatocellular carcinomas and some liver diseases. GLYAT was expressed in noncancerous hepatocytes but the protein disappeared in cancerous cells of all hepatocellular carcinomas. The repression of GLYAT expression and the degree of differentiation of the hepatocellular carcinoma showed no relationship. Hepatitis showed no repression of GLYAT and expression was normal. It is proposed that GLYAT can be an important molecule in the transition between carcinogenesis and differentiation of liver cells. They also suggested that GLYAT expression is regulated transcriptionally (Matsuo *et al.*, 2012).

### 2.8.4 Enzyme activity

The transfer of the acyl group from a CoA thioester of both aromatic as well as aliphatic acyl (C<sub>2</sub>-C<sub>10</sub>) groups is catalysed by an N-acyltransferase. Glycine N-acyltransferase (GLYAT) was found to show high specificity to the amino acid glycine but also to catalyse the transfer of the acyl group from the acyl-CoA to glycine's  $\alpha$ -amino (Schachter & Taggart, 1954b).

According to the literature the kinetic parameters for human GLYAT vary substantially with  $K_{Mapp}$  values (benzoyl-CoA) reported to be between 13  $\mu$ M and 57300  $\mu$ M,  $K_{Mapp}$  for glycine between 6.4 mM and 26.6 mM and the  $V_{max}$  values between 543 $\pm$ 21 nmol/min/mg and 17100 nmol/min/mg (Kelley & Vessey, 1994; Mawal & Qureshi, 1994; van der Westhuizen *et al.*, 2000; Matsuo *et al.*, 2012; van der Sluis *et al.*, 2013).  $K_{Mapp}$  is used when the kinetic parameters are determined with partially purified enzyme, if a purified enzyme are used for kinetic parameters it will be  $K_M$  values.

These parameters are summarised in Table 2.1. The parameters for the other acyl-CoAs that can be utilised are summarized in Table 2.2.

**Table 2.1: Summarised benzoyl-CoA and glycine kinetic parameters for human GLYAT.**

<b>Benzoyl-CoA (Km) (<math>\mu</math>M)</b>	<b>Glycine (Km) (mM)</b>	<b>Vmax (nmol/min/mg)</b>	<b>Reference</b>
67 $\pm$ 5	6.5 $\pm$ 1	N/A	Kelley and Vessey (1994)
57900	N/A	17100	Mawal and Qureshi (1994)
13.0	6.4	543 $\pm$ 21	van der Westhuizen <i>et al.</i> (2000)
209	26.6	807	Matsuo <i>et al.</i> (2012)
38 $\pm$ 4	N/A	1230 $\pm$ 60	van der Sluis <i>et al.</i> (2013)
49 $\pm$ 13	20 $\pm$ 4	157 $\pm$ 22	van der Sluis <i>et al.</i> (2017)

**Table 2.2: Summarised parameters of the other acyl-CoAs for human GLYAT.**

<b>Acyl-CoA</b>	<b>Km (<math>\mu</math>M)</b>	<b>Glycine (Km) (mM)</b>	<b>Vmax (nmol/min/mg)</b>	<b>Reference</b>
Salicyl-CoA	83700	N/A	10100	Mawal and Qureshi (1994)
Isovaleryl-CoA	12400	N/A	7640	Mawal and Qureshi (1994)
	672 $\pm$ 164	523 $\pm$ 206	13.4 $\pm$ 4.4	Gregersen <i>et al.</i> (1986)
Octanoyl-CoA	198000	N/A	3300	Mawal and Qureshi (1994)
	322 $\pm$ 87	770 $\pm$ 110	7.7 $\pm$ 0.5	Gregersen <i>et al.</i> (1986)
Butyryl-CoA	2400 $\pm$ 880	970 $\pm$ 210	29.5 $\pm$ 3.7	Gregersen <i>et al.</i> (1986)
Hexanoyl-CoA	2680 $\pm$ 770	1150 $\pm$ 210	26.5 $\pm$ 5.8	Gregersen <i>et al.</i> (1986)
Decanoyl-CoA	2408 $\pm$ 887	690 $\pm$ 210	20.4 $\pm$ 3.3	Gregersen <i>et al.</i> (1986)

### 2.8.4.1 Substrate specificity

Nandi *et al.*, (1979) showed that in bovine liver the acyl-CoA substrate will bind first and then the addition of an amino acid will follow before the CoA will dissociate, followed by the final step with the release of the peptide to form the product (Nandi *et al.*, 1979). The preferred acyl-CoA is benzoyl-CoA with a low  $K_m$  and high  $V_{max}$  which suggests a high affinity and reactivity for GLYAT (Bartlett & Gompertz, 1974a). Salicyl-CoA and some aliphatic acyl-CoAs can also serve as acyl-CoAs with glycine. Other amino acids, such as glutamine, alanine, glutamic acid and asparagine can also serve at low concentrations, with GLYAT stereospecific to some L forms (Nandi *et al.*, 1979; van der Westhuizen *et al.*, 2000). Matsuo *et al.*, (2012) demonstrated that the catalysis of benzoyl-CoA and glycine is more favourable over phenylacetyl-CoA for human GLYAT. These results suggest that human GLYAT is a typical aralkyl transferase (Matsuo *et al.*, 2012). The substrate specificity for both the acyl-CoA and the acyl acceptor are summarised in Table 2.3.

**Table 2.3: The substrate specificity for both the acyl-CoA and the acyl acceptor for human GLYAT**

Preference by GLYAT	Acyl acceptor	Preference by GLYAT	Acyl-CoA (Acyl donor)
1	Glycine	1	Benzoyl-CoA
2	Alanine	2	Salicyl-CoA
3	L-Glutamine	3	Isovaleryl-CoA
4	L-Asparagine	4	Butyryl-CoA
5	Glutamic acid	5	Octanoyl-CoA
		6	Propionyl-CoA
		7	Acetyl-CoA

(Mawal and Qureshi 1994)

### 2.8.4.2 Inhibitors of the glycine conjugation pathway

GLYAT is inhibited by phenylacetyl-CoA and indoleacetyl-CoA, these are the substrates for the closely related phenylacetyltransferase (Webster *et al.*, 1976; Nandi *et al.*, 1979). Formation of salicyl-CoA is inhibited by benzoic acid *in vivo* and *in vitro* by bovine ACSM (Vessey *et al.*, 1996;

Knights & Minors, 2012). Ethanol was established to have no effect on hippuric acid elimination but it inhibited benzoyl-CoA conjugation, where it had no effect on salicylurate excretion or of the formation of salicyl-CoA (Levy, 1979). KCl was found to be an inhibitory substance for glycination of benzoyl-CoA (Kelley & Vessey, 1994).

A significant discovery made by Schachter and Taggart was that glycine conjugation is inhibited by hippurate and CoASH, the products formed from this pathway, showing product inhibition. Hippurate as well as the CoASH are proposed to be non-competitive inhibitors. The inhibition of hippurate and CoASH were not influenced by the concentrations of glycine or benzoyl-CoA. Non-competitive inhibition occurs when both the substrate and the inhibitor bind to the enzyme simultaneously leading to no reaction taking place (Schachter & Taggart, 1954b).

#### **2.8.4.3 Enzyme kinetic mechanism of GLYAT**

Most previous studies on the enzyme kinetic mechanism of GLYAT assumed human GLYAT will follow sequential Bi-Bi mechanism like the bovine GLYAT. The human GLYAT enzyme kinetic mechanism has not yet been investigated enough (Kelley & Vessey, 1994; Mawal & Qureshi, 1994; van der Westhuizen *et al.*, 2000; Matsuo *et al.*, 2012; van der Sluis *et al.*, 2013). The limitation with doing kinetic studies on human GLYAT so far was the fact that enzyme was only partially purified or when using a mitochondrial lysate from liver tissue the variant was unknown. Van der Sluis *et al.* (2017) did the first study on a purified recombinant human GLYAT enzyme. They determined both bi-substrate kinetics and a kinetic mechanism on this purified sample. Their findings reported an equal goodness of fit for both the ping-pong mechanism and random Bi-Bi mechanism (van der Sluis *et al.*, 2017).

There are sulfotransferase enzymes (SULT) that when their enzyme kinetic mechanism were studied over a small concentration range, Michaelis-Menten kinetics were observed. As the concentration range became wider, cooperativity was exhibited (James MO., 2014). The  $K_{0.5}$  values for sigmoidal enzymes will vary at different concentrations of substrate. Sigmoidal enzymes are cooperative enzymes where a specific substrate concentration will result in  $V_{max}$ . The  $K_{0.5}$  value (the value of half of  $V_{max}$ ) will thus change as the substrate concentration changes (Bhagavan and Ha, 2015). This might explain why the  $K_m$  values for the GLYAT enzyme reported in the literature varies so immensely (Palmer & Bonner, 2007). Van der Sluis *et al.* (2017) raised the question that if the Michaelis-Menten mechanism is not the correct mechanism to apply to human GLYAT, might GLYAT not exhibit the same kinetic mechanism as some SULT enzymes?

#### 2.8.4.4 Possible general enzyme kinetic mechanisms

##### 2.8.4.4.1 Cooperativity

Thermodynamic or the kinetic properties of a system can be impacted by cooperativity. Thermodynamic effects of cooperativity are seen by a modification in the ligand binding affinity. Kinetic cooperativity is seen as a variation from hyperbolic kinetics in the rate response of an enzyme. Monomer enzymes with single ligand-binding sites are completely kinetic in basis, resulting in the Michaelis-Menten equation to be inadequate to define the rate upon varying substrate concentration (Porter & Miller, 2012).

The Hill coefficient ( $n$ ) is a measure of the cooperativity that an enzyme displays with respect to a substrate. More than one binding site on an enzyme can lead to the possibility of interaction between the binding sites during the process of binding and is termed *cooperativity*. The slope ( $n$ ) determines the steepness of the graph and this is an indication of the cooperativity. Cooperativity is reflected by the value of  $n$ . Positive cooperativity ( $n > 1$ ) is the binding of a substrate resulting in the increase of the affinity for the remaining substrates, similar or different, to bind to the enzyme. Usually *n more than one* is an indication that there is more than one binding site on the enzyme. Negative cooperativity ( $n < 1$ ) occurs as the binding of one substrate to the protein will decrease the affinity for the other substrates, similar or different, to bind. Non-cooperativity ( $n = 1$ ) is independent substrate binding; the substrates will bind to the enzyme regardless of one another (Palmer & Bonner, 2007; PhysiologyWeb, 2013). The Hill equation describes systems that are thermodynamic cooperative in terms of *n describing* the number of binding sites. As such, it is not possible to use the Hill coefficient for cooperativity with monomeric enzymes with single ligand-binding site (Porter & Miller, 2012).

##### 2.8.4.4.2 Possible mechanisms for monomeric enzymes

Cooperativity is frequently connected with protein conformational changes in monomeric enzymes with single ligand-binding site. Cooperativity in monomeric enzymes causes slow substrate-induced modifications in the enzyme structure that will inhibit substrate binding from achieving equilibrium in a certain amount of time of the catalytic turnover (Cornish-Bowden & Cárdenas, 1987).

##### 2.8.4.4.3 Mnemonic Model

The mnemonical model is based on monomeric cooperativity that depends on two forms of the existing enzyme. These two forms are different in stability as well as slow in isomerization. Richard, Meunier and Buc was the first to construct this model to help explain why monomeric

enzymes display non-hyperbolic kinetics. Frieden and Rabin proposed that an enzyme's conformation after product release could differ from the initial enzyme state. In 1970, Whitehead first proposed that an enzyme could have the ability to "recall" or "remember" the substrate-induced conformation. After a very short period of time the enzyme will "forget" the previous interaction (Cornish-Bowden & Cárdenas, 1987).

The mnemonic model contains two different species: the low affinity state ( $E^*$ ) and high affinity state ( $E$ ). Absence of substrate will favour the low-affinity state. When a substrate binds to an enzyme, a conformational transition to a ligand state will be induced. More than one substrate can bind to the enzyme in a fixed order. Cooperativity to one substrate is expected, usually the first substrate, followed by Michaelis-Menten for the second substrate. After the chemical reaction is complete and a product dissociates the enzyme will be in the high affinity state ( $E$ ). With high substrate concentrations, the high affinity state can quickly bind another substrate and another catalytic reaction will take place instead of the slowly realised low-affinity conformation. The enzyme in the high affinity state "remembers" the conformation leading to an increase in product formation. With lower substrate concentrations the rate in which a substrate binds to the enzyme is too long causing the enzyme to relax to the original low affinity conformation (Cornish-Bowden & Cárdenas, 1987; Porter & Miller, 2012). The binding of the substrate enzyme complex is not balanced, thus leading to cooperativity. The cooperativity is determined by the rate of the forward constants and not the rate of the balance for the two binding reactions (Cornish-Bowden & Cárdenas, 1987).

$$\frac{\text{rate constant for binding } E}{\text{rate constant for binding } E^*} > 1 = \text{positive cooperativity}; < 1 = \text{netative cooperativity}$$

#### 2.8.4.4 LIST (Ligand-induced Slow Transition)

Neet and Cardens were responsible for the development of the LIST model. This model is comparable to the mnemonic model. The LIST mechanism has two distinct enzyme conformations,  $E^*$  and  $E$ . Between the two enzyme species, an established equilibrium in the absence of substrate is already evident. Different affinities for each conformation exist for the substrate: the amount of substrate present will control the balance between these two states. Slow transition can involve two processes, isomerization or association-dissociation. The interconversion between the two conformations must be slower than the turnover, this prevents equilibrium during substrate association. This model assumes that each conformation can undergo a catalytic cycle, the resultant steady state velocity is the amount of the rates for the different two catalytic cycles (Porter & Miller, 2012).

#### 2.8.4.4.5 Random substrate addition

Ferdinand first worked on this model and it was further developed by Petterson. They wanted to explain the sigmoidal kinetics for the enzyme glucokinase. This model relies exclusively on random order binding unlike mnemonic and the LIST models. The random order does not rely on enzyme conformational changes or slow interconversion rates. The random addition of a substrate can result in cooperative behaviour. This happens when one of the substrates addition pathways are kinetically favoured. The less favourable pathway will contribute insignificantly to the reaction velocity (steady-state), providing a mechanism that has a non-productive intermediate ES complex that can accumulate. With abundant substrate and suitable rate constants, the condition can cause deviations from Michaelis-Menten kinetics (Porter & Miller, 2012).

#### 2.8.4.4.6 What kinetic model is exhibited by GLYAT?

Preliminary studies by van der Sluis *et al.* (2017) concluded that the Hill slope for glycine and varying benzoyl-CoA concentrations resulted in a sigmoidal enzyme mechanism being observed with positive cooperativity. The opposite occurred when the Hill slope for benzoyl-CoA was determined and glycine concentrations were varied. With glycine concentrations smaller and/or equal to 5mM a sigmoidal enzyme mechanism was observed with positive cooperativity. As soon as the glycine concentration increased the data exhibited the Michaelis-Menten mechanism (van der Sluis *et al.*, 2017).

These results can be used to explain why the enzyme kinetic parameters for GLYAT are not consistent. Previous studies that determined the kinetic parameters, used glycine concentration that was above 5mM, this resulted in Michaelis-Menten kinetics (Kelley & Vessey, 1994; Mawal & Qureshi, 1994; van der Westhuizen *et al.*, 2000). The recombinant wild-type (S<sub>156</sub>) variant was used to determine the kinetic parameters and very high benzoyl-CoA concentrations were used (Matsuo *et al.*, 2012).

These findings made it clear that the kinetic mechanism for GLYAT needs to be further evaluated, different haplotypes and different substrate concentrations must be used to be able to completely characterise this complex enzyme.

## 2.9 Problem statement

The problem with the increase in additives in food causes a bigger problem in the body as too much sodium benzoate is ingested. High levels of benzoic acid (10mg/kg body weight) can cause slight skin irritation and irritation of the digestive mucus (FAO/WHO, 2000; del Olmo *et al.*, 2017). GLYAT is the enzyme in the liver mitochondria responsible for conjugating benzoyl-CoA with

glycine to form hippurate, a product that can easily be excreted in urine. Interindividual variation in the *GLYAT* gene has an effect on the GLYAT enzyme. The SNPs can influence the relative enzyme activity and substrate specificity (van der Sluis *et al.*, 2013; van der Sluis *et al.*, 2013). If the enzyme activity is too low and the enzyme cannot change substrate into product fast enough, there will be a benzoate/benzoyl-CoA build-up in the body, this is toxic to the body. This study was conducted in order to determine the influence three haplotypes have on the GLYAT relative enzyme activity. The bi-substrate kinetics were tested to determine a kinetic model for GLYAT and substrate specificity was determined. This will help us better understand the enzyme and how the different haplotypes influences all the kinetic parameters.

## CHAPTER 3: CONSTRUCTION, EXPRESSION AND PURIFICATION OF GLYAT VARIANTS

### Introduction

The objective for this section of the study was to construct, express and purify the S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub> recombinant GLYAT variants. Glycerol stocks of the pGro7 chaperone, Origami cells, as well as the S<sub>156</sub>/pET32a(+) and the S<sub>156</sub>C<sub>199</sub>/pET32a(+) GLYAT variants in Origami/pGro7 cells were already available in the laboratory. The T<sub>17</sub>S<sub>156</sub> variant was constructed, expressed and purified. All three variants contained His-tags for western blotting verification.



### Materials and methods:

#### 3.1 Stock solutions

All stock solutions were prepared with molecular biology grade water. Sterile glycerol (80%, v/v) was prepared by adding glycerol to deionised water, followed by sterilisation by autoclaving for 15 minutes. The stock solution was stored at room temperature.

Glycerol stocks were prepared for long term storage by mixing 800 µl of the cells with 200 µl sterile 80% (v/v) glycerol in a microcentrifuge tube and freezing the stocks at -80°C.

#### 3.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation and visualisation of DNA samples. The DNA samples were separated on 1 % (w/v) agarose gels, with 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, and 1 mM EDTA, pH 8.5). The electrophoresis runs were performed at a constant voltage of 80V for 60 minutes, unless otherwise specified. The Syngene G:BOX F3 Fluorescence gel documentation system was used in conjunction with the GeneSys image acquisition software program (Syngene, Cambridge, UK). Ethidium bromide (EtBr) was used for visualisation of the DNA fragments. The O'GeneRuler 1kb DNA Ladder Mix (#SM 1173) (100-10,000 bp) was used as a size marker.

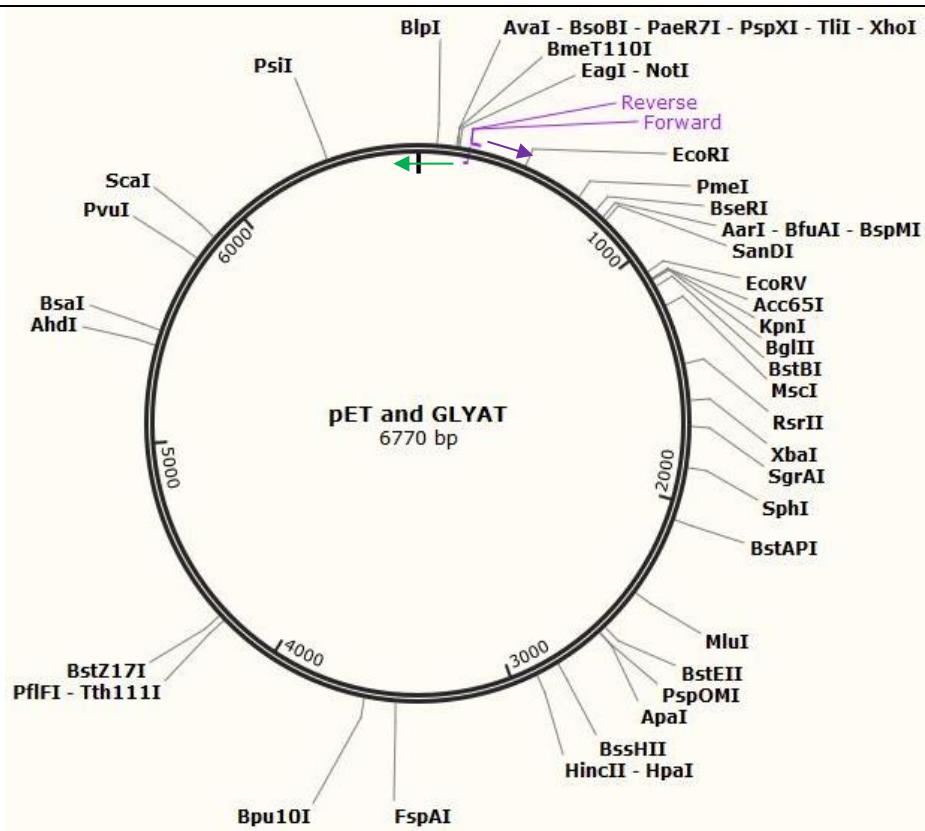
### 3.3 Constructing the T<sub>17</sub>S<sub>156</sub> variant

#### 3.3.1 Introduction of the T<sub>17</sub>S<sub>156</sub> mutation

The T<sub>17</sub>S<sub>156</sub> variant was not available in our laboratory and as such a PCR reaction was used to introduce the S<sub>17</sub>T mutation, a synonymous mutation (TCC → ACC) at position 17 of the GLYAT DNA insert. The plasmid used as DNA template already contained the S<sub>156</sub> mutation, which is the change of AAT → AGT at position 156. The primers used to introduce TCC → ACC mutation within the S<sub>156</sub>/pET32(+) construct (see Table 1) was already available in the laboratory and was designed by van der Sluis *et al.* (2013). The entire plasmid will be amplified including the insert. The primers were designed to extend in opposite directions of the plasmid and this resulted in a linearised plasmid containing the two mutations. See Figure 3.1 as an example. The Forward primer contains the DNA change, this will be on the outside of the double DNA strand and the reverse primer are on the inside.

**Table 3.1: PCR Primers for the T<sub>17</sub>S<sub>156</sub> plasmid**

Sequence	
Forward	5'-cag atg ctg gag aaa <b>Acc</b> ttg agg aag agc-3'
Reverse	5'-ttg caa ggt gcc cag atg ctg-3'



**Figure 3.1:** A representation of the pET32a(+) vector containing the GLYAT S156 mutation, with the use of PCR and the forward primer and reverse primer the T17S156 mutation was introduced. The direction of the forward primer is indicated with the purple arrow and the reverse with the green.

### 3.3.2 Polymerase chain reaction (PCR) optimisation of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linear fragment.

The Phusion High-Fidelity DNA Polymerase from ThermoScientific was used for the PCR reaction. Phusion DNA polymerase holds 5' → 3' DNA polymerase activity as well as 3' → 5' exonuclease activity which yields blunt end amplification products.

The reaction mixture for the PCR was set up according to the manufacturer's instructions, consisting of: 10 µl 5x Phusion HE buffer; 4 µl dNTPs; 2.5 µl Forward primer (10mM); 2.5 µl Reverse primer (10mM); 2 µl Template DNA [89.4ng/µL]; 2.5 µl DMSO, 1 µl Phusion Hot Start II DNA polymerase, 25.5 µl nuclease free water was added to a final volume of 50 µl.

The PCR reaction was optimised by varying the annealing temperatures (65,2°C; 67,2°C; 69,9°C and 71,9°C), the conditions can be seen in Table 3.2. The extension time was determined by using 15 seconds per 1000bp, the final extension time is 6x the extension time which is 8 minutes and 40 seconds.

**Table 3.2:** PCR reaction mix optimisation.

Cycle step	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	98	1:00	1
Denaturation	98	0:15	35
Annealing	65,2 - 71,9	0:30	
Extension	72	1:40	
Final Extension	72	8:40	1
Hold	4	∞	∞

The PCR amplification was evaluated by running the samples on a 1% agarose electrophoresis gel (Section 3.2.)

The PCR reaction was repeated with the optimal annealing temperature (69.9°C) determined to obtain the conditions shown in Table 3.3.

**Table 3.3:** PCR cycle conditions.

Cycle step	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	98	1:00	1
Denaturation	98	0:15	35
Annealing	69,9	0:30	
Extension	72	1:40	
Final Extension	72	8:40	1
Hold	4	∞	∞

### 3.3.3 Gel extraction of the desired T<sub>17</sub>S<sub>156</sub> fragment

The PCR product (T<sub>17</sub>S<sub>156</sub>/pET32a(+)) linear fragment) was loaded onto an agarose gel as described in Section 3.2, S<sub>156</sub>/pET32a(+) plasmid was used as a positive control. The NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel, Düren, Germany; #1502/001) was used for the gel extraction. A 2mL tube was weighed beforehand. The amplified linear vector band was cut out of the agarose gel, the strip was then placed into the pre-weighed 2mL tube. 200 µl buffer (NTI) per 100 mg of gel was added to the fragment. The sample was incubated at 50°C for 10 minutes then vortexed for 2 minutes to solubilise the agarose. The sample solution was loaded onto a spin column and centrifuged at 11 000 x g for 30 seconds at room temperature. This allowed the solution to pass through the silica membrane resulting in DNA to bind to the membrane in the presence of the buffer NTI. The membrane was washed with 700µL NT buffer by centrifugation at 10000 x g for

30 seconds at room temperature. The wash step was repeated twice. The membrane was then centrifuged at 11 000 x g for 2 minutes at room temperature to dry the membrane. The DNA was eluted with 15µL of nuclease-free water, centrifuged at 11 000 x g for 2 minutes at room temperature, this step was repeated. All steps were performed at room temperature.

An agarose gel was prepared as described in Section 3.2 and the samples were loaded for verification of the successful extraction of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) fragment.

#### 3.3.4 Determining the concentration of T<sub>17</sub>S<sub>156</sub>/pET32a(+)

The concentration of DNA was determined by using spectrophotometry. The NanoDrop One (ThermoFisher Scientific) was the instrument used to determine DNA concentrations. Beer-Lamberts law's principles is used in this method. dsDNA absorbs light at 260nm, leading to the A<sub>260</sub>/A<sub>280</sub> ratio. This ratio reveals that if samples are contaminated with proteins because proteins absorb light at 280nm. A A<sub>260</sub>/A<sub>280</sub> ration of 1.8 is seen as normal, anything below is seen as contaminated.

#### 3.3.5 Ligation of the linear T<sub>17</sub>S<sub>156</sub>/pET32a(+) vector

The gel extracted linearised T<sub>17</sub>S<sub>156</sub>/pET32a(+) vector needed to be recirculated by the use of the Rapid DNA Ligation Kit (ThermoFisher Scientific). T<sub>17</sub>S<sub>156</sub>/pET32a(+) vector was diluted (50ng) and the reaction could be set up according to the manufacturer's instructions, which is 1µL of the T4 DNA ligase; 10µL 5x Rapid Ligation Buffer; 1µL linearized T<sub>17</sub>S<sub>156</sub>/pET32a(+) vector and 38µL water to a final volume of 50µL. The sample was vortexed after which it was incubated at 22°C for 5 minutes.

#### 3.3.6 Transformation of the ligated T<sub>17</sub>S<sub>156</sub>/pET32a(+) fragment into DH5α cells

The ligated T<sub>17</sub>S<sub>156</sub>/pET32a(+) fragment was transformed into commercially competent DH5α cells (Invitrogen). 50µL DH5α cells were thawed on ice. 1µL of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) ligated fragment was added to the cells and incubated on ice for 30 minutes. The cells were then heat shocked in a 42°C water bath for 20 seconds; this was done without shaking the tube. The tube containing the cells was placed on ice for 2 minutes after which 950µL pre-warmed S.O.C (Super Optimal broth with catabolite repression, composition consisted of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose) medium was added to the tube. This was incubated for 1 hour at 37°C with shaking at 225 rpm. Nutrient agar plates containing 50 µg/mL Carbenicillin were made and two different volumes (100µl and 150µl) were plated out onto the plates to ensure that one plate had well-spaced colonies. The plates were incubated overnight at 37°C.

### 3.3.7 Screening and verification of transformed bacterial colonies

Six colonies were selected. These colonies were used to inoculate 10mL Terrific Broth (TB) medium (12% tryptone (w/v), 24% yeast extract, (w/v), 9.4% Potassium phosphate dibasic (w/v) and 2.2% Potassium phosphate monobasic (w/v)) containing 50 µg/ml Carbinicillin for selection of bacteria containing the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmids. The same six colonies were used to inoculate 10 ml Luria-Bertani (LB) medium, (1% tryptone (w/v), 0.5% yeast extract, (w/v), 1% NaCl (w/v)) containing 50 µg/ml Carbinicillin. The different mediums (TB and LB) were used to determine in which medium the colonies would grow best since GLYAT is toxic to the bacteria. Glycine was added to both mediums to a final concentration of 0.5%. The samples were incubated overnight at 37°C shaking at 170 rpm.

### 3.3.8 Plasmid extraction of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid.

To extract the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid for sequencing the PureYield Midiprep system (Promega) was used. Bacterial cell cultures were grown from the previously described six colonies. 250mL TB and LB medium was used to ensure optimal plasmid extraction. The colonies were inoculated in both TB and LB medium containing 50µg/mL Carbinicillin and glycine to a final concentration of 0.5%. The samples were incubated overnight at 37°C with shaking at 180rpm for 13 hours. The OD<sub>600</sub> never reached 2 and anything above 1.4 was accepted.

The samples were centrifuged at 5000 x g at room temperature for 10 minutes, the supernatant was discarded and the pellet dried by draining the tubes on paper towels. The pellet was resuspended in 6mL Cell Resuspension solution. 6mL cell lysis solution was added and the tubes were inverted 3-5 times, the samples were then incubated at room temperature for 3 minutes. Neutralizing solution, 10mL, was added to each sample to lyse the cells. The tubes were inverted multiple times. Centrifugation at 15 000 x g for 15 minutes at room temperature to pellet the cellular debris followed.

The PureYield Clearing Column was stacked upon a ClearYield Binding Column. The lysate was then decanted into the Cleaning Column and a vacuum was applied. The DNA will then bind to the membrane of the Binding Column. Endotoxin Removal wash was added to the Binding Column after all the lysate passed through. Column Wash was lastly added to the Binding Column, The washing buffer contains ethanol and as such it was important to dry the membrane before DNA could be eluted. After the membrane was dry, 400µL Nuclease-free water was used to elute the desired plasmid; this volume of Nuclease-free water ensured a higher plasmid yield.

To analyse whether the plasmid isolation was successful, the plasmids from the six colonies were loaded onto a 1% agarose gel (Section 3.2.)

### 3.3.9 Restriction enzyme digestion of the isolated T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid to verify the size of the insert

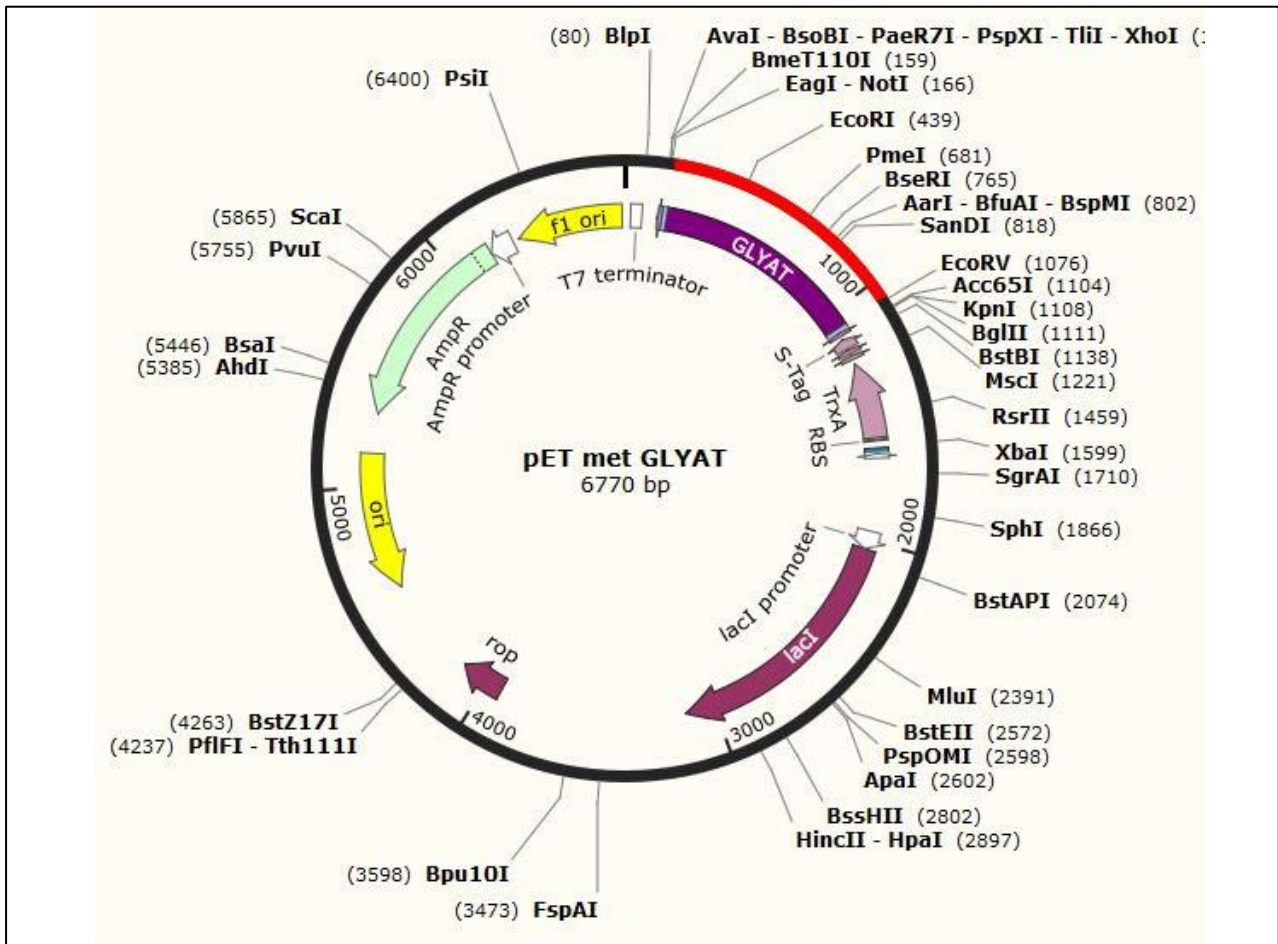
This step was done to verify excision of the expected size fragment. The inserts were excised from the pET32a(+) plasmids by using restriction enzymes, BamHI and HindIII. Table 3.4 summarises the use of one restriction enzyme for a linear plasmid with the insert intact, and the use of two restriction enzymes to excise the insert from the plasmid. These conditions were recommended by the Fermentas DoubleDigest software program which calculated the desired parameters. The reactions were made up as seen in Table 3.4. The tubes were gently mixed and spun down. The samples were then incubated for 20 minutes in a heating block at 37°C followed by agarose gel electrophoresis as described in section 3.2. The agarose gel electrophoresis was performed to separate the pET32a(+) plasmids and the desired insert. Table 3.5 shows exactly where both BamHI and HindIII cutting sites are. Figure 3.2 shows on the pET32a(+) vector map where the restriction enzymes are located.

**Table 3.4:** Restriction enzyme reactions

	T <sub>17</sub> S <sub>156</sub> /pET32a(+)Col 1 - 6 Linearized	T <sub>17</sub> S <sub>156</sub> /pET32a(+)Col 1-6 Double digestion
10 x Fast Digest green buffer	2 µL	2 µL
Digested enzyme (BamHI)	1µL	1µL
Double digest (HindIII)	-	2µL
DNA	Up to 1 µg	Up to 2 µg
Nuclease free water	Fill up to a final volume of 20 µL	Fill up to a final volume of 20 µL
Total	20µL	20µL

**Table 3.5:** Enzyme summary

BamHI	5'...G↓GATCC...3'
HindIII	5'...A↓AGCTT...3'



**Figure 3.2: pET32a(+) vector map.** The vector map (pET32a(+)) with GLYAT, inserted at the restriction sites BamHI and HindIII.

### 3.3.10 Sanger sequencing of plasmids

Sanger sequencing was performed on the samples to determine the successful insertion of the mutation. Before the samples were sent for sequencing their DNA concentrations were determined using the NanoDrop One, as described in Section 3.3.4. Some of the samples had a concentration lower than 100ng/μL. These samples were concentrated using the SpeedVac concentrator (Cat. No.ISS110FS-115) (Thermo Scientific, Waltham, MA, USA).

All six samples were sent for sequencing at the Central Analytical Facility located at Stellenbosch University. The sequencing primers are summarised in Table 3.6. The DNA chromatograms received by the Analytical Facility were viewed and aligned using the BioEdit program.

**Table 3.6:** Sequence primers

Primer	Recognition sequence
S-Tag Promoter	5'- GAACGCCAGCACATGGAC - 3'
T7 Terminator	5'- GCTAGTTATTGCTCAGCGG - 3'

### 3.4 Expression and verification of the variants

#### 3.4.1 Co-transformation of Origami/pGro7 cells with the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid

Chemically competent Origami cells already containing the pGro7 plasmids (already available in the laboratory) were used for transformation with the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid. The competent Origami cells, containing the pGro7 plasmids, were thawed completely on wet ice for approximately 20 minutes. 100 ng of the plasmid DNA (1 µl) was then added to the cells and incubated on ice for 1 hour. 800 µl transformation storage buffer (TSBG) (1.6% Peptone (w/v), 1.0% Yeast Extract (w/v), 0.5% NaCl (w/v), 10.0% PEG (w/v), 5.0% DMSO (w/v), 1M MgCl<sub>2</sub>, 1M MgSO<sub>4</sub>, 20mM glucose) was then added to the cells. The cells were then incubated at 37°C shaking at 225rpm for 1 hour.

Nutrient agar plates were prepared with Carbinicillin added to a final concentration of 50µg/ml and Chloramphenicol added to a final concentration of 20 µg/ml. After the transformation, 50µL, 100µl and 150µL of the sample was streaked out on different agar plates. The plates were incubated overnight at 37°C.

In 50 ml TB medium (with 50 µg/ml carbinicillin and 20 µg/ml chloramphenicol for selection) a single colony was then inoculated from the overnight plates containing the co-transformed colonies and incubated overnight at 37°C with shaking at 225 rpm.

Glycerol stocks were prepared and stored at -80 °C. These were then used for inoculation of cultures for the expression steps.

#### 3.4.2 Protein expression

All three GLYAT variants needed to be expressed. Van der Sluis *et al.* (2013) successfully optimised a protocol to express GLYAT as a soluble and enzymatically active protein. The method used also included the use of chaperones (pGro7).

The expression medium was prepared using 2% bacto-tryptone (w/v), 1.25% yeast extract (w/v), 0.625% NaCl (w/v), 0.5% Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O (w/v), 0.1% KH<sub>2</sub>PO<sub>4</sub> (w/v), and 0.2% (w/v) glucose and glycine to a final concentration of 0.5%. Inoculation was done from glycerol stocks in 10 ml of the expression medium. The cultures were incubated overnight at 37 °C, shaking at 180 rpm.

The cells were then harvested by centrifugation (4000 x g, 5 minutes, 4 °C). The harvested cells were resuspended in 100 ml of the same expression medium, an optical density value of < 0.5 was necessary.

The cultures were incubated at 28 °C for 24 hours with shaking at 200 rpm for the expression of the proteins (van der Sluis et al., 2013). The use of L-Arabinose induced the chaperones, 2 mg/ml L-Arabinose was added to the medium before use. No isopropyl β-D-1-thiogalactopyranoside (IPTG) was necessary as the proteins were expressing through 'leaky' expression.

#### 3.4.3 Cell lysis of the bacterial cells

A 100 ml lysis buffer consisting of 50 mM NaH<sub>2</sub>PO<sub>4</sub> and 300 mM NaCl was prepared (van der Sluis et al., 2013). The pH was then set to pH 8 and filter sterilised using a sterile 0.8/0.2 µm Acrodisc PF Syringe Filter. After sterilisation 10% (v/v) Glycerol, 1% (v/v) Triton X-100 and 10ul Lysonase (for every 10 ml buffer) was added.

After the incubation period of 24 hours, 28°C, 100 ml of the expression medium was used. The cells were harvested by centrifugation, 4000 x g for 15 minutes at 4°C. The pelleted cells were resuspended in 10 ml of the lysis buffer and was incubated on ice for 10 minutes. The lysates were passed through a 22G needle five times for further cell disruption. The lysates were centrifuged at 14 000 x g, 25 minutes, 4°C to get rid of all cell debris. The supernatant, which served as the soluble fraction, was saved for further SDS-PAGE analyses.

#### 3.4.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to monitor protein expression and purification. A 10% TGX Stain-free Fast Cast Acrylamide Kit from BioRad was used (Cat#161-0183). The SDS-PAGE gels were made according to the manufactures protocol received with the kit.

Protein samples were prepared by mixing 15 µl of the sample with 5 µl of 4 x Laemmli Sample Buffer (Cat#161-0747). The samples were boiled for 5 minutes at 98°C, after which they were immediately used for the SDS-PAGE analysis. Unless otherwise specified, 15 µl was loaded onto the gel for electrophoresis. Precision Plus Protein Dual Color Standards #161-0374 (10 kDa to 250 kDa) was used as a molecular size marker. The gel was then placed in the gel chamber with

1x TGS buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.6). For electrophoresis, the voltage was 130V for 60 minutes.

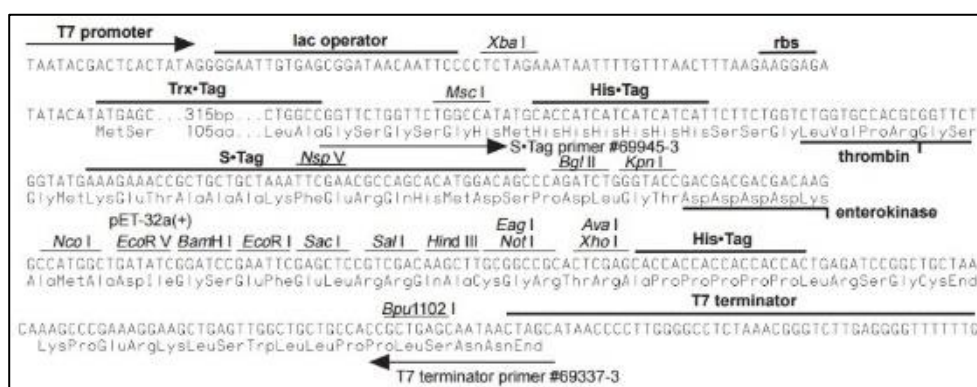
Thereafter, the gel was removed from the glass plates and, for the visualisation of the protein bands, placed into a container with Coomassie gel staining solution (0.25g Coomassie Brilliant Blue in 90ml methanol:H<sub>2</sub>O (1:1 v/v)) for approximately 2 hours, shaking at 50 rpm. The SDS-PAGE gel, was then removed from the Coomassie gel staining solution and rinsed with water to remove excess stain. The SDS-PAGE gel was placed in a container filled with destaining solution (50% (v/v) methanol, 10% (v/v) acetic acid) while shaking at 60 rpm 25°C, changing the solution every half an hour or until the blue stain was removed from the gel. The ChemiDoc MP Imaging system was used together with the ImageLab 5.2.1 software program to view the gel.

### 3.4.5 Purification using nickle affinity columns

The poly-His tags fused to the GLYAT variants N-termini was used for successful purification, see Figure 3.3. For purification purposes a nickel-affinity chromatography was used. The Protino Ni-TED 2000 column kit was used to obtain the purified protein. The protocol was followed as described in the manual (Rev 06).

4ml LEW buffer was added to the column for equilibration. The binding step consisted of adding the cleared lysates to pass through the column which was followed by a wash step to clean the membrane by adding 2 X 5 ml LEW buffer to the column. The proteins were eluted with 3 ml EB buffer (Macherey-Nagel Inc., Bethlehem, PA).

Samples were taken from each of the steps that were run on a SDS-PAGE for verification of the purified protein. The protocol for SDS-PAGE is explained in Section 3.4.4.



**Figure 3.3: pET32a(+) expression cassette.** The vector map shows the Trx-tag followed by the His-tag and the S-tag that GLYAT was expressed with making the enzyme approximately 56kDa.

### 3.4.6 Western blot verification of the three GLYAT proteins

This was done for verification of the three variant GLYAT proteins. All three proteins were expressed with N-terminal Trx-His-tags, see Figure 3.3. The His-tags are six Histidine residues (HHHHHH) that were recognised by the antibodies.

A SDS-PAGE gel was run as described in Section 3.4.4, the gel was removed from the glass plates but was not stained by Coomassie. The Precision Plus Protein WesternC Blotting Standards (#1610376) ladder was used which is specifically made for western blotting. The blotting sandwich was assembled as described in the Trans-Blot Turbo Transfer System Transfer Pack (Bio-rad #170-4156). The blotting sandwich contained the bottom iron reservoir stack, the PVDF membrane, the gel and the top iron reservoir stack. The sandwich was placed into the ChemiDoc station from Bio-rad; the standard option was used for the western blotting, 30 min, 1.0 A and 25V.

After the blotting was completed the membrane was placed for 1 and a half hours in blocking buffer which consisted of TBS (Tris-buffered saline) with 1% Casein on a rocking platform. The membrane was washed after that time in washing buffer (TBS and 0,1% Tween20) for 5 minutes, this step was repeated three times.

The membrane was probed with the Precision Plus Strep Tactin-HRP conjugated antibody for the Precision Plus Protein WesternC ladder at a dilution of 1:10 000 (diluted with TBS and 1% Casein). The membrane was left at room temperature for 1 hour on a rocking platform. The membrane was washed again with washing buffer, repeated 3 times.

The primary antibody was specific for the His tags on the proteins, 6x-His Tag Monoclonal Antibody (HIS.H8), HRP (MA1-21315-HRP). The membrane was probed with the 6x-His tag antibody at a dilution of 1:1000 (TBS and 1% Casein). This was left overnight at 4°C on a rocking platform. The membrane was washed with washing buffer. This step was repeated three times followed by the final washing solution (TBS) for 5 minutes.

For visualisation, the Clarity Western ECL substrate mixture (#1705060) was used along with the ChemiDoc station and the Image Lab V. 5.2.1 software. The kit contains two components that were mixed in a ratio of 1:1 (Peroxide:Luminol/enhancer solution) as instructed in the manual. The mixture was added onto the membrane and left for 5 minutes after which the visualisation took place.

### 3.4.7 Determining protein concentration

It was important to determine the concentrations of the different proteins before any analysis could be determined. To identify the concentrations, the Qubit Protein Assay Kit (pre diluted SBA

standards based) from Molecular Probes were used. The Qubit Protein Assay Kit uses specific dyes that bind to the target protein, resulting in the fluorescence of the samples.

The Qubit comes with a standard protocol which will be described shortly. A working solution was prepared as well as a set of standards. For the working solution 5 $\mu$ L reagent and 995 $\mu$ L buffer were combined. The standards were made up by mixing, in special Qubit tubes, 10 $\mu$ L of each standard given with 190 $\mu$ L of the working solution that was prepared. The Qubit system was calibrated using these standards.

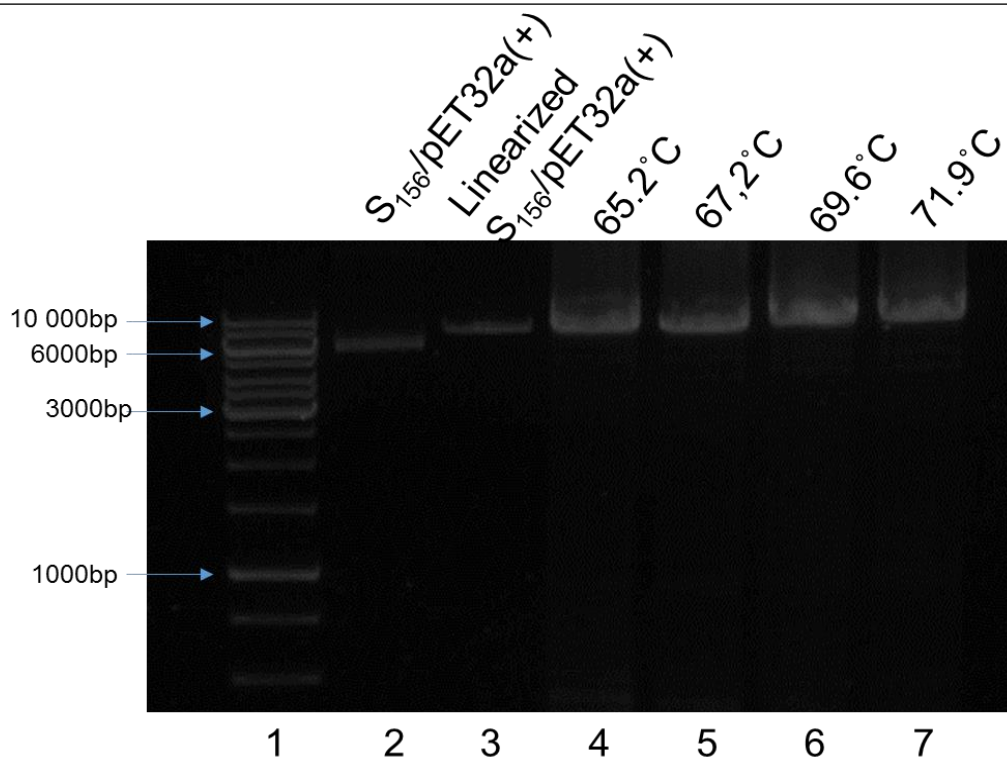
The samples were prepared by combining 1 $\mu$ L of each protein sample with 199 $\mu$ L of the previously prepared working solution, this was also completed in the special Qubit tubes. The tubes containing the samples were mixed by vortex and left to incubate at room temperature for 15 minutes. The protein content was measured, each sample was measured three times to determine repeatability.

### 3.5 Results and Discussion

#### 3.5.1 Polymerase chain reaction (PCR) optimisation of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linear fragment

The Phusion High-Fidelity DNA Polymerase from ThermoScientific was used for the PCR reaction. Phusion DNA polymerase holds 5' → 3' DNA polymerase activity as well as 3' → 5' exonuclease activity which yields blunt end amplification products. The protocol was performed as described in Section 3.3.2. The T<sub>17</sub> (TCC → ACC) mutation was introduced into the pET32a(+) vector already containing the S<sub>156</sub> mutation (AAT → AGT). Figure 3.4 shows the different PCR annealing temperatures that were tested. To determine which temperature is sufficient to produce one fragment without any nonspecific amplification. From the results it can be seen that the annealing temperatures that were used all showed very good results, 69.6°C was used as the optimal annealing temperature to amplify the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linear fragment as it showed the highest yield.

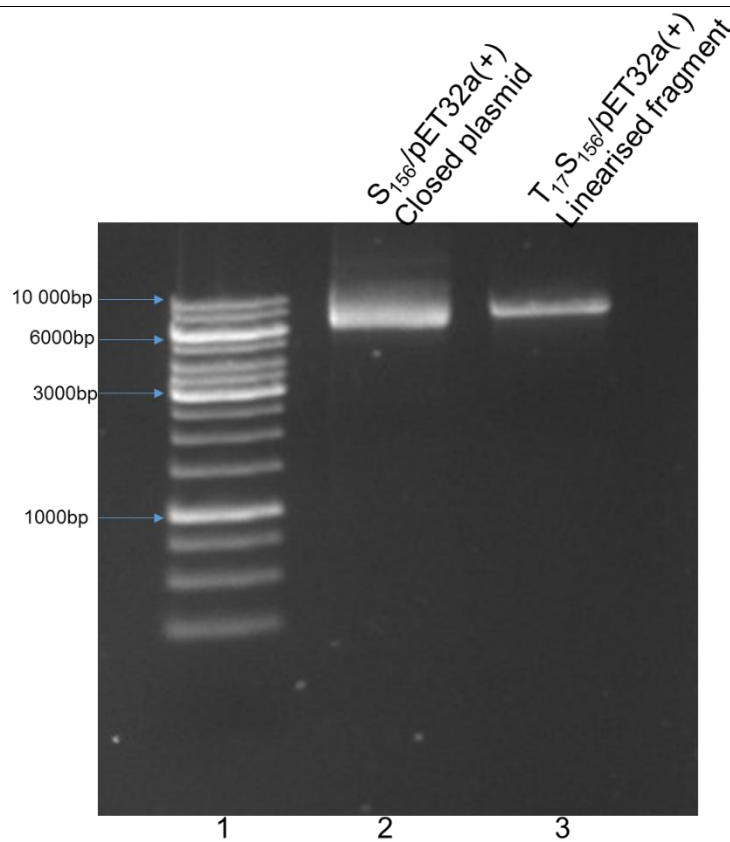
In lane 1 the O'Gene ruler was loaded as a molecular marker followed by the closed S<sub>156</sub>/pET32a(+) (lane 2), the linearized S<sub>156</sub>/pET32a(+) (lane 3) and then the products of the PCR using the different annealing temperatures (lane 4-7). The size of T<sub>17</sub>S<sub>156</sub>/pET32a(+) is 6771bp, the plasmid is 5900bp and GLYAT 891bp.



**Figure 3.4: PCR optimization.** Lane 1: The O'gene ruler as molecular marker, 2: S<sub>156</sub>/pET32a(+) as control, 3: linearized S<sub>156</sub>/pET32a(+), 4: 65.2°C, 5: 67,2°C, 6: 69.6°C and 7: 71.9°C

### 3.5.2 Gel extraction of desired T<sub>17</sub>S<sub>156</sub> fragment

There is plasmid left in after the PCR reaction was performed because it was used as the template for the PCR. Therefore if you do not do gel extraction, you will get preferential transformation of the closed plasmid. To ensure that only the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linearised fragment was used in the subsequent ligation and transformation reaction, gel extraction was carried out - this is where the linearised fragment is cut out of the gel. Gel electrophoresis was done to verify the successful extraction of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linearised fragment. The S<sub>156</sub>/pET32a(+) vector was used as a control as seen in figure 3.5 the vector in lane 2 is the S<sub>156</sub>/pET32a(+) closed plasmid and is also lower than 3 which is the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linearised fragment. The closed plasmid will migrate through the gel faster than the linearised fragment, this is why the S<sub>156</sub>/pET32a(+) closed plasmid is lower on the gel compared to the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linearised fragment even though they are the same base pairs.



**Figure 3.5: 1% Agarose gel of the linear PCR T<sub>17</sub>S<sub>156</sub>/pET32a(+) vector.** 1: O'Gene ruler as molecular marker, 2: The circular S<sub>156</sub>/pET32a(+) and 3: The linear T<sub>17</sub>S<sub>156</sub>/pET32a(+)

### 3.5.3 Ligation of linear T<sub>17</sub>S<sub>156</sub>/pET32a(+) vector

To close the T<sub>17</sub>S<sub>156</sub>/pET32a(+) linear fragment a ligation reaction was done. The T<sub>17</sub>S<sub>156</sub>/pET32a(+) linearised fragment was ligated using the Rapid DNA Ligation Kit (ThermoFisher). The protocol was described in Section 3.3.4.

### 3.5.4 Screening and verification of the transformed bacterial colonies

Six bacterial colonies were selected and inoculated in order to isolate plasmid DNA (Section 3.3.5). In the DH5 $\alpha$  cells there is leaky expression of GLYAT, this is toxic to the bacteria as all the glycine is used; with the additional 0.5% glycine added the growth rate improves. Two different mediums were tried for inoculation purposes, Luria – Bertani (LB) and Terrific Broth (TB). In the TB medium the bacterial colonies resulted in better growth compare to the LB medium. In Table 3.7 the difference can clearly be seen in terms of OD<sub>600</sub>. All the colonies except colony three had better growth. Colony one had almost twice as much growth in the TB medium compared to the LB medium.

**Table 3.7:** OD<sub>600</sub> of TB and LB after 13 hours

Colony	LB medium and glycine	TB medium and glycine
1	1.381	1.624*
2	1.427	1.500*
3	1.464*	1.440
4	1.436	1.497*
5	1.475	1.547*
6	1.526	1.657*

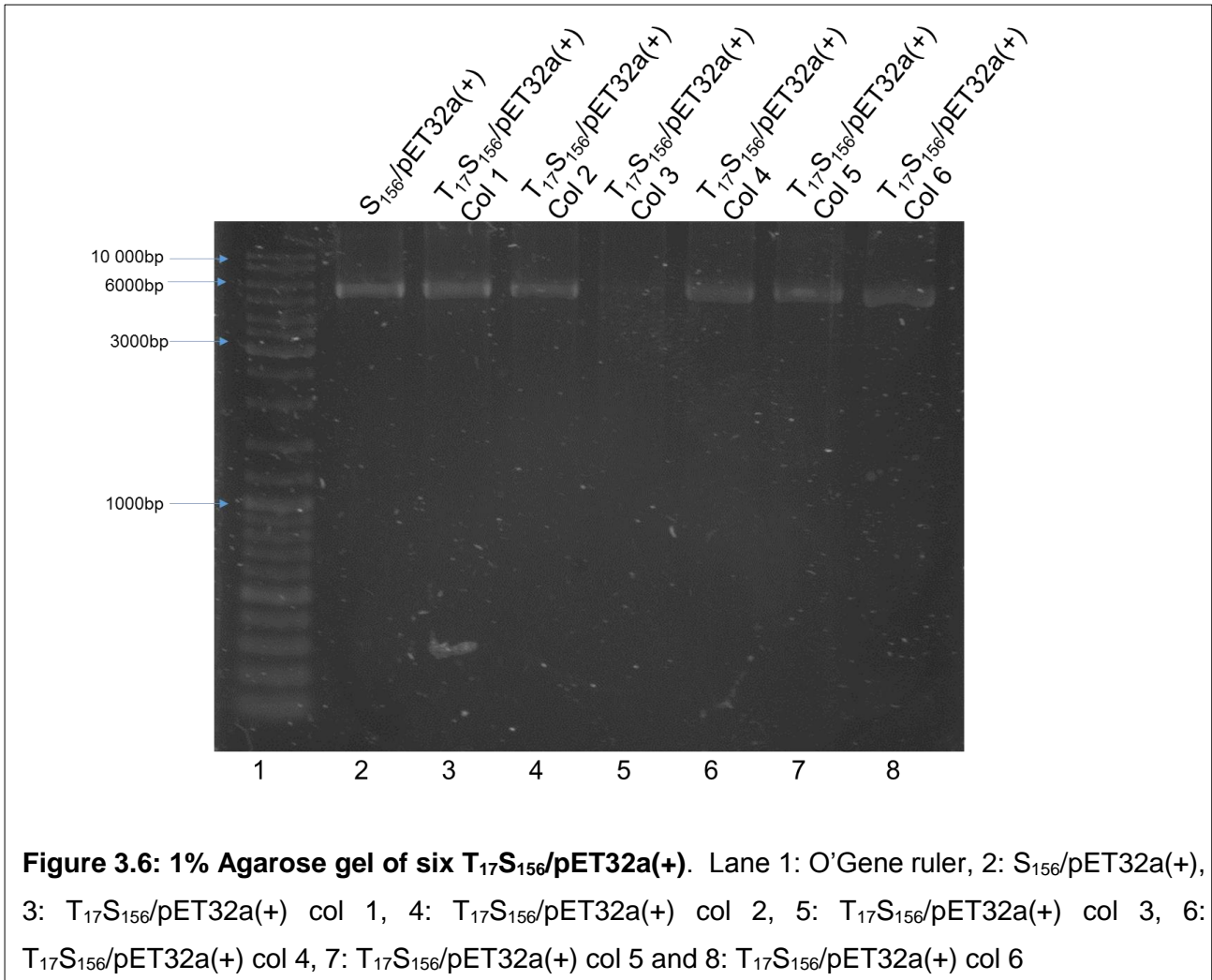
The TB broth contains more tryptone and yeast extract, this might be why the bacteria grew better when compared to the LB medium. The samples with the highest OD values were used further in the study (indicated by a \*). These samples were also used to make glycerol stocks for long term storage.

### 3.5.5 Plasmid extraction of the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid

The six different samples were used for plasmid extraction (midi-prep) to isolate the T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid. The plasmid extractions were done as explained in Section 3.3.8. Gel electrophoresis was prepared to verify the possible T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmids, see Figure 3.6. The O'Gene ruler was loaded as a molecular marker and the S<sub>156</sub>/pET32a(+) was used as a control.

The concentration of the six different colonies were determined using the NanoDrop One, see Section 3.3.4. Table 3.8 shows the different concentrations for the six different colonies. Colonies 2 - 4 had an initial low concentration. For sequencing reaction a minimum concentration of 100ng/μL for the plasmids are needed. The initial concentrations for colonies 2 – 4 were below a 100ng/μL. The SpeedVac was used to obtain higher concentrations. Table 3.8 only shows the higher concentration levels of colonies 2 – 4 and the initial concentrations of colonies 1, 5 and 6. Colonies 1.5 and 6 had DNA concentration above 100ng/μL and therefor the SpeedVac was not needed on them.

Colony 3 did not show a clearly visible band, the concentration was correct (above 100ng/μL). It could be possible that the sample was injected to quickly and resulted in the sample mixing with the running buffer. Colonies 1, 2, 4, 5 and 6 illustrates successful plasmid extraction. Colony 3 is seen very lightly.

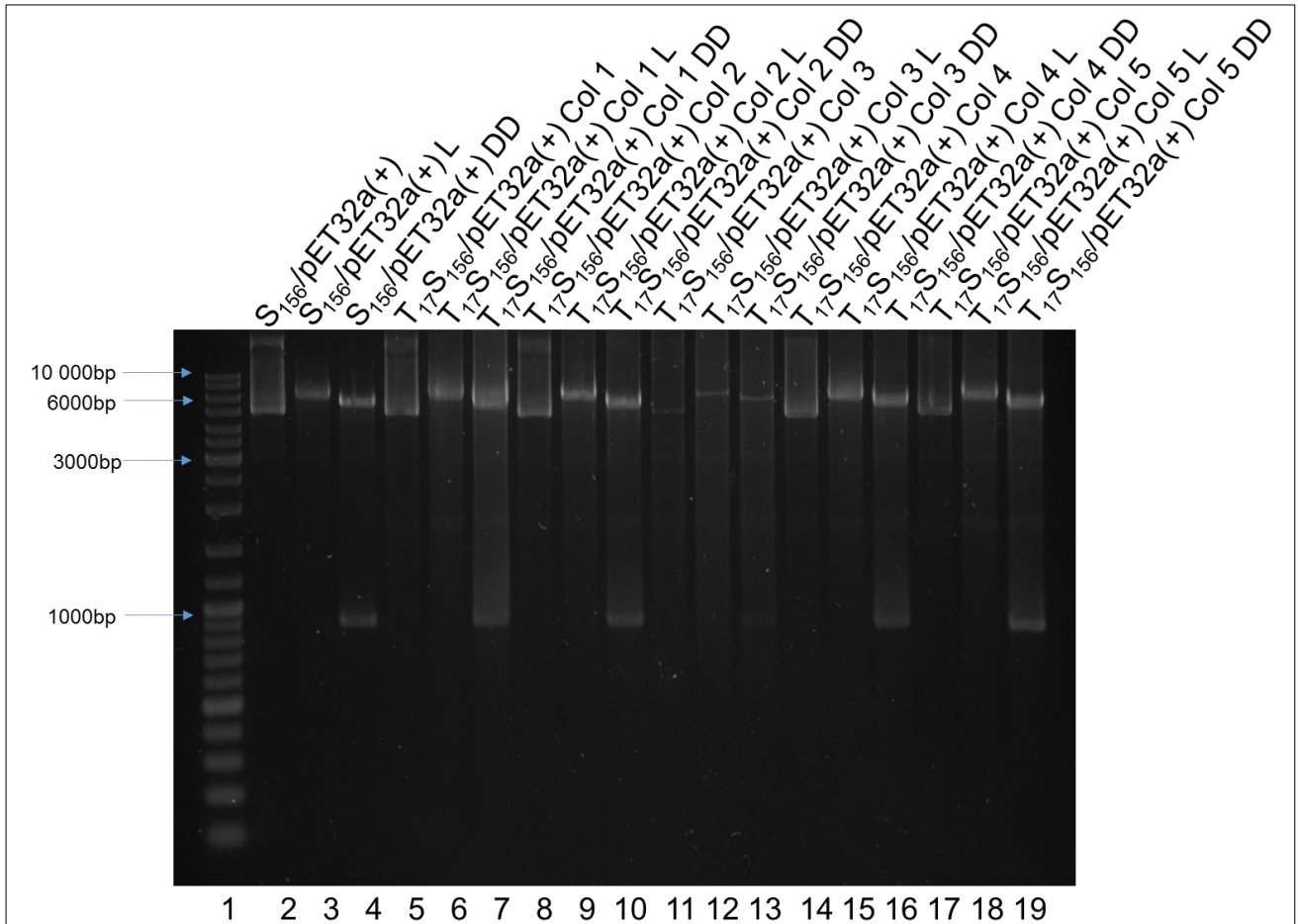


**Table 3.8:** Concentrations of the different colonies

Colony	Concentration (ng/μl)	260/280
1	157.4	1.82
2	168.3	1.81
3	104.8	1.80
4	157.1	1.83
5	113.9	1.81
6	132.6	1.84

### 3.5.6 Restriction enzyme digestion of the isolated T<sub>17</sub>S<sub>156</sub>/pET32a(+) plasmid to verify the size of insert

The insert and the plasmid were enzymatically digested for a verification step to see if the insert (GLYAT) and plasmid (pET32a(+)) have the same size respectively, described in Section 3.3.7. The results will be two different bands - the top band will be only the pET32a(+) plasmid and the lower band will be GLYAT (T<sub>17</sub>S<sub>156</sub>). The pET32a(+) plasmid is approximately 5880bp in size since BamHI and HindIII were the cutting sites and GLYAT is 891bp. In Figure 3.7 the O'Gene ruler was loaded as a molecular weight marker, the S<sub>156</sub>/pET32a(+) as a control and each of the six samples were linearized with the use of BamHI and then double digested with BamHI and HindIII. Colonies 1 - 5 had the insert at the correct place, 891bp. This shows successful verification for colonies 1 – 5 of the size of the GLYAT insert.



**Figure 3.7: 1% Agarose gel of five  $T_{17}S_{156}/pET32a(+)$  colonies.** Lane 1: O'Gene ruler, 2:  $S_{156}/pET32a(+)$ , 3:  $S_{156}/pET32a(+)$  Linearized, 4:  $S_{156}/pET32a(+)$  Double Digested, 5:  $T_{17}S_{156}/pET32a(+)$  col 1, 6:  $T_{17}S_{156}/pET32a(+)$  col 1 Linearized, 7:  $T_{17}S_{156}/pET32a(+)$  col 1 Double digested, 8:  $T_{17}S_{156}/pET32a(+)$  col 2, 9:  $T_{17}S_{156}/pET32a(+)$  col 2 Linearized, 10:  $T_{17}S_{156}/pET32a(+)$  col 2 Double digested, 11:  $T_{17}S_{156}/pET32a(+)$  col 3, 12:  $T_{17}S_{156}/pET32a(+)$  col 3 Linearized, 13:  $T_{17}S_{156}/pET32a(+)$  col 3 Double digested, 14:  $T_{17}S_{156}/pET32a(+)$  col 4, 15:  $T_{17}S_{156}/pET32a(+)$  col 4 Linearized, 16:  $T_{17}S_{156}/pET32a(+)$  col 4 Double digested, 17:  $T_{17}S_{156}/pET32a(+)$  col 5, 18:  $T_{17}S_{156}/pET32a(+)$  col 5 Linearized and 19:  $T_{17}S_{156}/pET32a(+)$  col 5 Double digested.

### 3.5.7 Sanger sequencing of plasmids

Sanger sequencing was done to determine the DNA sequence of the  $T_{17}S_{156}/pET32a(+)$  plasmid. The sequencing results confirmed that the  $T_{17}S_{156}$  *GLYAT* gene was successfully constructed. At position 17 (shown as purple) a serine codon (TCC) was changed to a threonine codon (ACC) and at position 156 (shown in green) an asparagine codon (AAT) was changed to a serine codon (AGT). These changes were made by site-directed mutagenesis.

Figure 3.8 shows the aligned sequences of the reference Wild-type *GLYAT* gene, the reference S<sub>17</sub>T *GLYAT* construct and then the sequence of the DNA made by the forward (S-tag) and reverse (T7 terminator) primers. The purple and green box shows the position where the SNPs were successfully introduced.

Wild-type	-----	-----	ATGATGTTAC
T17S156	-----	-----	ATGATGTTAC
T17S156_pET32_Koll_F_S-	CCATGGCTGA	TATCGGATCC	ATGATGTTAC
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	CATTGCAAGG	TGCCCAGATG	CTGCAGATGC
T17S156	CATTGCAAGG	TGCCCAGATG	CTGCAGATGC
T17S156_pET32_Koll_F_S-	CATTGCAAGG	TGCCCAGATG	CTGCAGATGC
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	TGGAGAAATC	CTTGAGGAAG	AGCCTCCCAG
T17S156	TGGAGAAAC	CTTGAGGAAG	AGCCTCCCAG
T17S156_pET32_Koll_F_S-	TGGAGAAAC	CTTGAGGAAG	AGCCTCCCAG
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	CATCCTTAAA	GGTTTATGGA	ACTGTCTTTC
T17S156	CATCCTTAAA	GGTTTATGGA	ACTGTCTTTC
T17S156_pET32_Koll_F_S-	CATCCTTAAA	GGTTTATGGA	ACTGTCTTTC
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	ACATAAACCA	TGGAAATCCA	TTCAATCTGA
T17S156	ACATAAACCA	TGGAAATCCA	TTCAATCTGA
T17S156_pET32_Koll_F_S-	ACATAAACCA	TGGAAATCCA	TTCAATCTGA
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	AGGCTGTGGT	GGACAAGTGG	CCTGATTTTA
T17S156	AGGCTGTGGT	GGACAAGTGG	CCTGATTTTA
T17S156_pET32_Koll_F_S-	AGGCTGTGGT	GGACAAGTGG	CCTGATTTTA
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	ATACAGTGGT	TGTCTGCCCT	CAGGAGCAGG
T17S156	ATACAGTGGT	TGTCTGCCCT	CAGGAGCAGG
T17S156_pET32_Koll_F_S-	ATACAGTGGT	TGTCTGCCCT	CAGGAGCAGG
T17S156_pET32_Koll_R_T7	-----	-----	-----
Wild-type	ATATGACAGA	TGACCTTGAT	CACTATACCA
T17S156	ATATGACAGA	TGACCTTGAT	CACTATACCA
T17S156_pET32_Koll_F_S-	ATATGACAGA	TGACCTTGAT	CACTATACCA
T17S156_pET32_Koll_R_T7	-----	-----AT	CACTATACCA
Wild-type	ATACTTACCA	AATCTACTCC	AAAGATCCCC
T17S156	ATACTTACCA	AATCTACTCC	AAAGATCCCC
T17S156_pET32_Koll_F_S-	ATACTTACCA	AATCTACTCC	AAAGATCCCC
T17S156_pET32_Koll_R_T7	ATACTTACCA	AATCTACTCC	AAAGATCCCC
Wild-type	AAAACTGTCA	GGAATTCCTT	GGATCACCAG
T17S156	AAAACTGTCA	GGAATTCCTT	GGATCACCAG
T17S156_pET32_Koll_F_S-	AAAACTGTCA	GGAATTCCTT	GGATCACCAG
T17S156_pET32_Koll_R_T7	AAAACTGTCA	GGAATTCCTT	GGATCACCAG
Wild-type	AACTCATCAA	CTGGAAACAG	CATTTACAGA
T17S156	AACTCATCAA	CTGGAAACAG	CATTTACAGA
T17S156_pET32_Koll_F_S-	AACTCATCAA	CTGGAAACAG	CATTTACAGA
T17S156_pET32_Koll_R_T7	AACTCATCAA	CTGGAAACAG	CATTTACAGA
Wild-type	TTCAAAGTTC	ACAGCCTAGC	CTGAATGAGG
T17S156	TTCAAAGTTC	ACAGCCTAGC	CTGAATGAGG
T17S156_pET32_Koll_F_S-	TTCAAAGTTC	ACAGCCTAGC	CTGAATGAGG
T17S156_pET32_Koll_R_T7	TTCAAAGTTC	ACAGCCTAGC	CTGAATGAGG
Wild-type	CTATACAAAA	TCTTGCAGCC	ATTAAGTCCT
T17S156	CTATACAAAA	TCTTGCAGCC	ATTAAGTCCT
T17S156_pET32_Koll_F_S-	CTATACAAAA	TCTTGCAGCC	ATTAAGTCCT
T17S156_pET32_Koll_R_T7	CTATACAAAA	TCTTGCAGCC	ATTAAGTCCT
Wild-type	TCAAAGTCAA	ACAAACACAA	CGCATTCTCT
T17S156	TCAAAGTCAA	ACAAACACAA	CGCATTCTCT
T17S156_pET32_Koll_F_S-	TCAAAGTCAA	ACAAACACAA	CGCATTCTCT
T17S156_pET32_Koll_R_T7	TCAAAGTCAA	ACAAACACAA	CGCATTCTCT

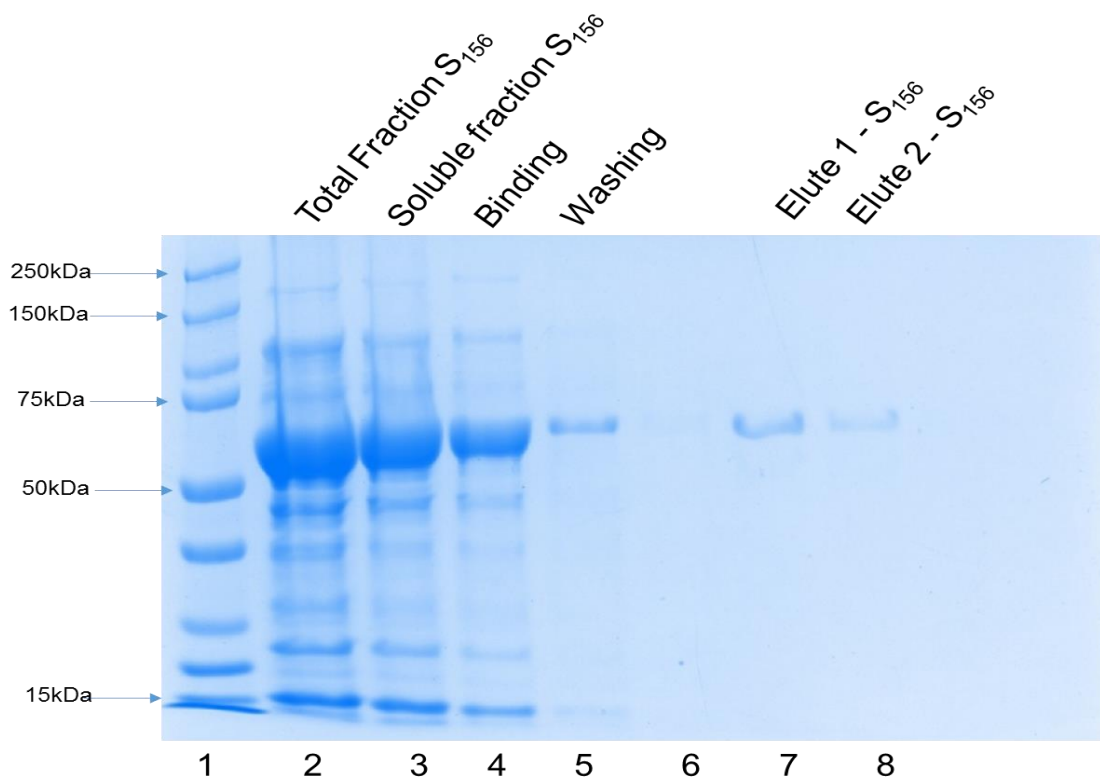


### 3.5.8 Expression and purification of the S<sub>156</sub>/pET32a(+), T<sub>17</sub>S<sub>156</sub>/pET32a(+) and S<sub>156</sub>C<sub>199</sub>/pET32a(+) proteins

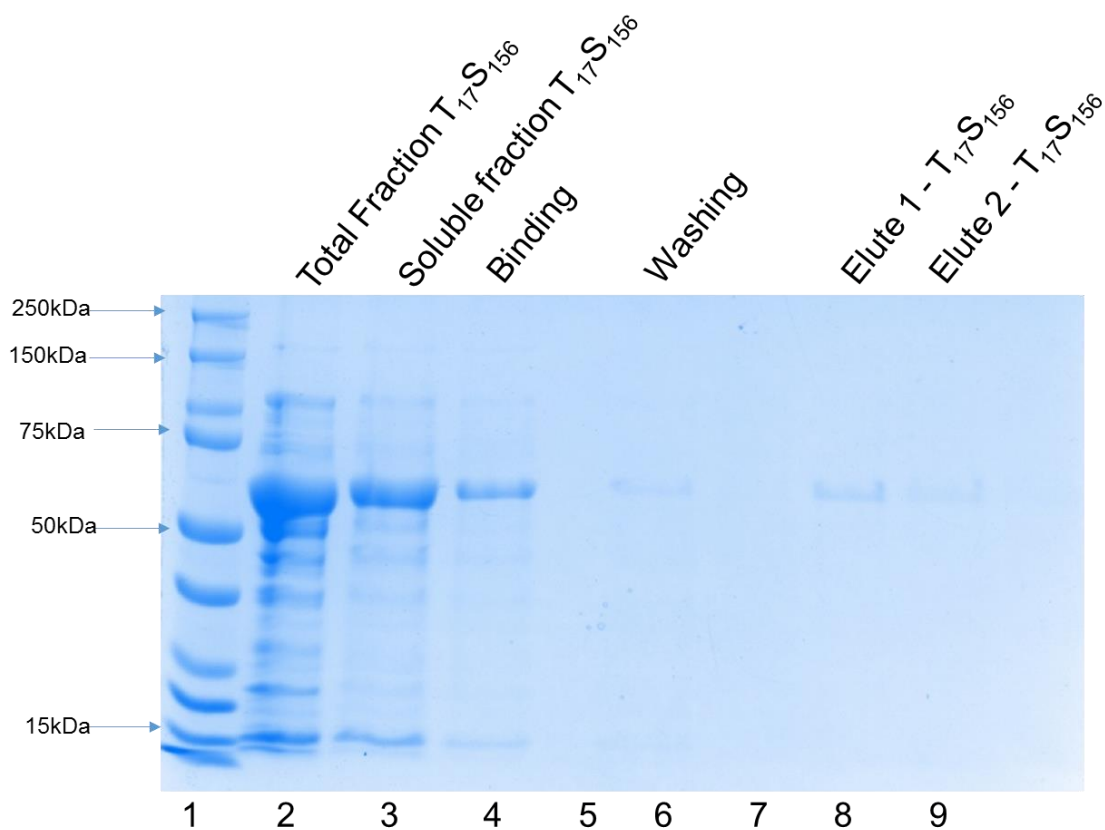
All three variants were expressed in Origami\_pGro7 cells as described in section 3.4.2 and 3.4.3. to determine the enzyme kinetic parameters. The pGro7 plasmid expresses the GroEL and GroES molecular chaperons which helped with folding the proteins. Both GroEL and GLYAT are at the same size (~52kDa). GLYAT is 891bp, this is approximately 32kDa. The Trx-His-tag fusion

is approximately 20kDa, adding up GLYAT to a final size of ~ 52kDa. The proteins were purified using nickel-affinity columns described in section 3.4.5.

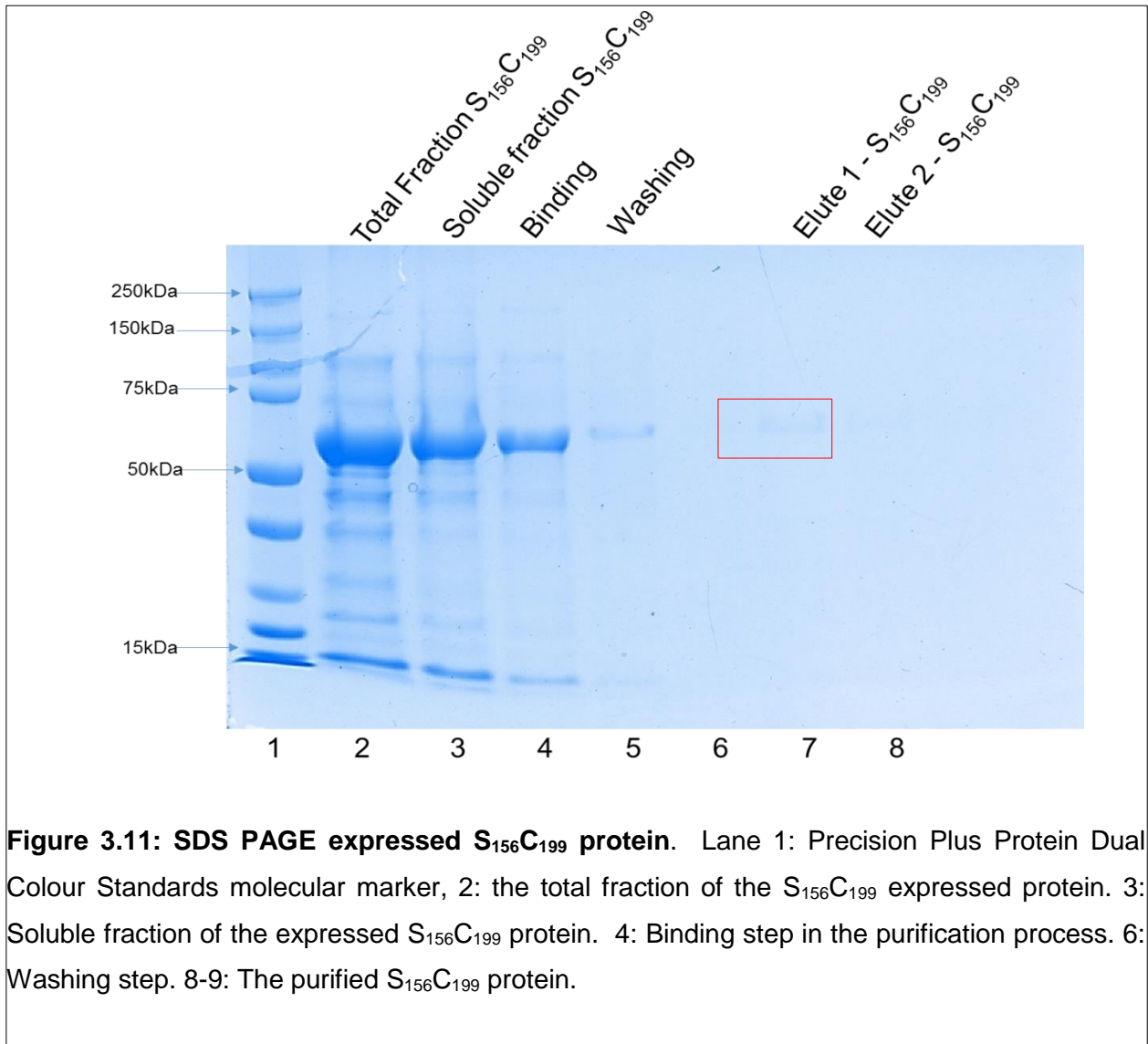
In Figures 3.9 to 3.11 it can be seen that all three proteins showed successful expression.  $S_{156}$  shows a higher level of expression when compared to the other two proteins after purification. This might be because  $S_{156}$  is more stable, has the highest enzyme activity and the highest haplotype frequency in the population. The  $T_{17}S_{156}$  showed expression that is comparable to the  $S_{156}$  protein but the  $S_{156}C_{199}$  protein had very low expression, see Figure 3.11 indicated by the red box. The  $S_{156}C_{199}$  protein might express so low because the  $R_{199}C$  variant is proposed to be located on an  $\alpha$ -helix elemental structure leading to the addition of a positive charge to the final charge of the protein, this extra charge might influence the folding of the protein. For all three variants the Precision Plus Protein Dual Colour Standards #161-0374 (10 kDa to 250 kDa) was used as a molecular size marker.



**Figure 3.9: SDS PAGE of expressed  $S_{156}$  protein.** Lane 1: Precision Plus Protein Dual Colour Standards molecular marker, 2: the total fraction of the  $S_{156}$  expressed protein. 3: Soluble fraction of the expressed  $S_{156}$  protein. 4: Binding step in the purification process. 5: Washing step. 7-8: The purified  $S_{156}$  protein.

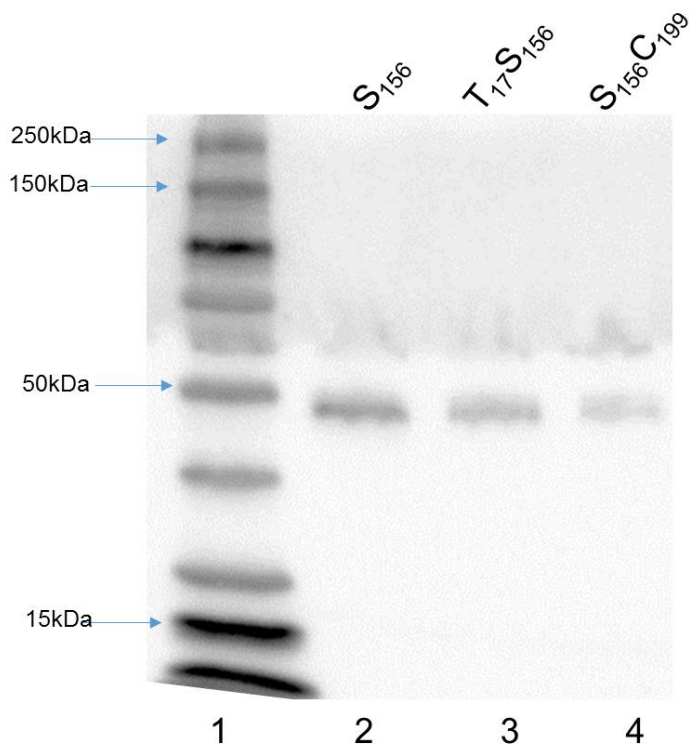


**Figure 3.10: SDS PAGE expressed T<sub>17</sub>S<sub>156</sub> protein.** Lane 1: Precision Plus Protein Dual Colour Standards molecular marker, 2: the total fraction of the T<sub>17</sub>S<sub>156</sub> expressed protein. 3: Soluble fraction of the expressed T<sub>17</sub>S<sub>156</sub> protein. 4: Binding step in the purification process. 6: Washing step. 8-9: The purified T<sub>17</sub>S<sub>156</sub> protein.



### 3.5.9 Western blot verification of the three GLYAT proteins

In order to verify that the purified proteins were the expressed GLYAT variants, a western blot was carried out. The GLYAT variants were all expressed with an N-terminal His tag that was recognised by the antibodies, described in section 3.4.6. The Precision Plus Protein Western Blotting Standard #161-0374 ladder was used that is also specifically made for western blotting. The ladder will bind to its own type of antibodies that will make the ladder visible on the western blotting membrane. As seen from Figure 3.12, all three proteins were successfully expressed at size ~52 kDa. S<sub>156</sub> shows the brightest band, the T<sub>17</sub>S<sub>156</sub> is slightly lighter than the S<sub>156</sub> variant and S<sub>156</sub>C<sub>199</sub> is very light. This corresponds with the expression of the different proteins. Figure 3.12 was over enhanced to ensure the visualization of the S<sub>156</sub>C<sub>199</sub> protein and as such a lot of background is visible.



**Figure 3.14: Western blot of the His tag purified proteins.** Lane 1: the molecular marker, 2: the S<sub>156</sub> expressed protein. 3: the expressed T<sub>17</sub>S<sub>1</sub> protein. 4: the expressed S<sub>156</sub>C<sub>199</sub> protein.

### 3.5.10 Determining protein concentrations

It was necessary to determine the concentration (described in 3.4.7) of the proteins before any kinetic work could be done. The concentrations are seen in Table 3.9. The protein concentrations were carried out in triplicate reaction for accuracy and repeatability. The average, standard deviation and the %CV were calculated. Any %CV value between 0 – 15% is acceptable (Whitmire *et al.*, 2011). All three proteins fall in that range.

**Table 3.9:** Protein concentrations

<b>Protein</b>	<b>Reaction</b>	<b>Concentration µg/mL</b>	<b>Concentration µg/µL</b>	<b>Average µg/µL</b>	<b>Standard deviation</b>	<b>%CV</b>
<b>S<sub>156</sub></b>	1	2.64	0.528	0.4906	0.042771	8.7
	2	2.22	0.444			
	3	2.50	0.500			
<b>T<sub>17</sub>S<sub>156</sub></b>	1	2.23	0.446	0.394	0.045797	11.60
	2	1.82	0.38			
	3	1.79	0.358			
<b>S<sub>156</sub>C<sub>199</sub></b>	1	2.18	0.436	0.411	0.02248	5.45
	2	2.03	0.406			
	3	1.96	0.392			

### 3.6 Summary

The aim of this part of the study was to express three GLYAT variants and to purify them. Both the S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub> constructs were already available in the laboratory. The T<sub>17</sub>S<sub>156</sub> needed to be constructed. This variant was constructed using site-directed mutagenesis, T<sub>17</sub>S<sub>156</sub> was sent for sanger sequencing to confirm the correct insert was in the pET32a(+) vector.

S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub> GLYAT variants were successfully expressed and purified. These variants were expressed in the Origami\_pGro7 expression system and then extracted using lysis buffer. The proteins were expressed with N-terminal Trx-His tags for purification. They were purified using nickel affinity columns. The purification was needed as the enzymes were used for enzyme kinetic parameters.

## CHAPTER 4: ENZYME KINETICS

According to previous studies conducted, the kinetic mechanism for GLYAT was assumed to follow a sequential Bi-Bi mechanism (Kelley & Vessey, 1994; Mawal & Qureshi, 1994; Matsuo *et al.*, 2012; van der Sluis *et al.*, 2013). Van der Westhuizen *et al.* (2002) proposed that the kinetic mechanism for GLYAT is sequential two substrate mechanism (van der Westhuisen *et al.*, 2002). The most recent study on GLYAT kinetics states that GLYAT showed sigmoidal enzyme kinetics that could be described using the Hill equation (van der Sluis *et al.*, 2017).

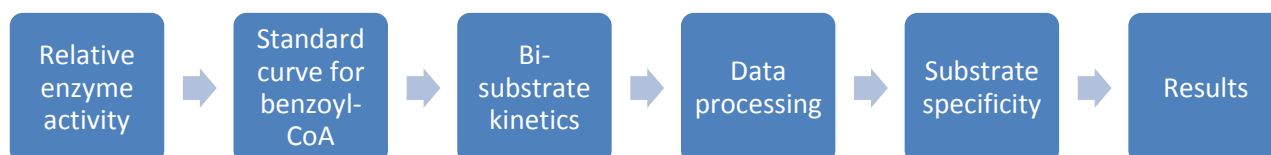
The limitation with doing kinetic studies on human GLYAT so far was the fact that enzyme was only partially purified or when using a mitochondrial lysate from liver tissue the variant was unknown. Van der Sluis *et al.* (2017) did the first study on a purified recombinant human GLYAT enzyme.

There are sulfotransferase enzymes (SULT) that, when their enzyme kinetic mechanism were studied over a small concentration range, showed Michaelis-Menten kinetics. As the concentration range became wider, cooperativity was exhibited (James MO., 2014). Van der Sluis *et al.* (2017) concluded that the Hill slope for glycine and varying benzoyl-CoA concentrations resulted in a sigmoidal enzyme mechanism being observed with positive cooperativity. When the Hill slope for benzoyl-CoA was determined and glycine concentrations were varying, with glycine concentrations smaller and/or equal to 5mM, a sigmoidal enzyme mechanism was observed with positive cooperativity. As soon as the glycine concentration increased the data exhibited Michaelis-Menten kinetics (der Sluis *et al.*, 2017).

Because of these limitations adding to the big variation in the kinetic parameters and the difference in enzyme kinetic mechanism the GLYAT enzyme needs to be classified further and more in depth studies must be done. The first objective of this part of the study was to confirm the sigmoidal enzyme mechanism identified in van der Sluis *et al.* (2017) using a wider range of substrate concentrations, benzoyl-CoA (0 – 200  $\mu$ M) and glycine (0 – 200mM). The second objective of this part of the study was to evaluate whether the different haplotypes (S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub>) can affect or alter the proposed kinetic mechanism. The three different enzymes were chosen because the S<sub>156</sub> variant (SNP) is the wild-type with the highest enzyme activity and the highest allele frequency. The S<sub>17</sub>T variant had a relative enzyme activity that is the third highest and the R<sub>199</sub>C variant had barely any enzyme activity. Previous studies evaluated the kinetic parameters of single SNPs but no kinetic data is available on any of the GLYAT haplotypes. This study will also aid in understanding the enzyme kinetics better in terms of the different haplotypes. See Table 4.1 as a summary of the data collected from previous studies.

**Table 4.1:** Summary of GLYAT Km and Vmax Values

Benzoyl-CoA (Km) ( $\mu$ M)	Glycine (Km) (mM)	Vmax (nmol/min/mg)	Recombinant or Homogenous extract from liver tissue	Reference
67 $\pm$ 5	6.5 $\pm$ 1	N/A	Homogenous extract from liver tissue – variant not known	Kelley and Vessey (1994)
57900	N/A	17100	Homogenous extract from liver tissue – variant not known	Mawal and Qureshi (1994)
13.0	6.4	543 $\pm$ 21	Homogenous extract from liver tissue – variant not known	van der Westhuizen <i>et al.</i> (2000)
209	26.6	807	Recombinant – variant not known	Matsuo <i>et al.</i> (2012)
38 $\pm$ 4	N/A	1230 $\pm$ 60	Recombinant (S <sub>156</sub> )	van der Sluis <i>et al.</i> (2013)
49 $\pm$ 13	20 $\pm$ 4	157 $\pm$ 22	Recombinant (S <sub>156</sub> )	van der Sluis <i>et al.</i> (2017)



## Materials and Methods

## 4.1 Stock solutions

All stock solutions were prepared with molecular grade water. The benzoyl-CoA stock solution was prepared with a stock concentration of 10 mM by dissolving 5mg in 575 $\mu$ L water, aliquoted and stored at -20°C. Glycine stock solution had a final concentration of 1M.

## 4.2 The relative enzyme activities of three GLYAT haplotypes

In 1959 George L. Ellman introduced 5,5'-dithio-bis-(2-nitrobenzoic acid), now known as DTNB. DTNB reacts with a free sulfhydryl group to produce a varied disulfide and 2-nitro-5-thiobenzoic acid (TNB) because DTNB is the conjugate base (R-S) of a free sulfhydryl group. This compound in a solution will yield an assessable yellow-coloured product when it reacts with sulfhydryls (Ellman, 1959). A colorimetric assay was used to determine the amount of products formed from benzoyl-CoA, glycine and the different GLYAT variants. This is a sufficient method for enzyme activity and kinetics. This method is based on glycine dependent release of CoASH which will give measurable results at 412nm (Kølvraa & Gregersen, 1986).

To verify that the purified GLYAT variants were active, an enzyme activity assay was conducted. This was also an indication of the level of enzyme activity of each of the different haplotypes (S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub>) using saturating substrate concentrations.

The enzyme assay consisted of a master mix that was composed of 0.1mM DTNB, 25mM TrisCl (pH 8), 100 $\mu$ M benzoyl-CoA, 200mM glycine and nuclease-free water to a final volume of 100 $\mu$ L. In a 96-well plate. 2 $\mu$ g of the three enzyme variants were added separately to each well, subsequently 100 $\mu$ L of the master mix was added, the 96-well plate was briefly shaken. The BioTek plate reader, together with the Gen5 software, was used for the analysis. The reactions were measured for 6 minutes at 37°C with the absorbance measured every 15 seconds at 412nm. The negative control used, did not contain any glycine.

## 4.3 Standard curve of benzoyl-CoA

The gradient of the standard curve graph was used during the kinetic data processing. The  $r^2$  value is also an indication to evaluate how well the data correlates with the linear regression. To construct the data points for the standard curve, an end-point assay for each data point was performed in triplicate using different benzoyl-CoA concentrations (20 $\mu$ M, 40 $\mu$ M, 80 $\mu$ M, 100 $\mu$ M, 120 $\mu$ M, 140 $\mu$ M, 160 $\mu$ M and 200 $\mu$ M) and saturating glycine concentrations of 200mM. The reaction was allowed to continue until a plateau was reached. This is an indication that the reaction has reached equilibrium. The S<sub>156</sub> variant was used as this is the most active enzyme and also the wild-type.

A master mix solution was prepared consisting of 0.01 mM DTNB (diluted in 1 M TrisHCl), 200mM glycine and nuclease-free water. The final volume of each well was 200  $\mu$ L.

Into a 96-well plate the purified protein (2 $\mu$ g) was added in triplicate. 20 $\mu$ L of the different benzoyl-CoA concentrations was rapidly added followed by the master mix. This was done with a multi-channel pipette. The plate was gently shaken for 25 minutes at 37°C and absorbance was measured at 412nm by the BioTek plate reader and Gen5 software.

#### **4.4 Bi-substrate kinetics**

The kinetic parameters for the three GLYAT proteins were determined by using an enzyme assay, with both substrate concentrations being varied. The benzoyl-CoA concentration was varied from 0 - 200 $\mu$ M while keeping the glycine concentrations constant. The glycine concentration were varied from 0 – 200mM whilst keeping the benzoyl-CoA concentrations constant. See appendix (Table 4.2) for an example of a 96-well plate layout with benzoyl-CoA concentration of 20 $\mu$ M and varying glycine concentrations.

A master mix was prepared for more consistent data. The master mix consisted of 0.01mM DTNB, diluted Tris-Cl (25mM) and nuclease-free water. The benzoyl-CoA was prepared in a separate 5 mL tube, the concentrations used for this study were 0  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 40  $\mu$ M, 60  $\mu$ M, 80  $\mu$ M, 100  $\mu$ M, 120  $\mu$ M, 140  $\mu$ M, 160  $\mu$ M, 180  $\mu$ M and 200  $\mu$ M. The different glycine concentrations were pipetted into a clean 96-well plate, 40  $\mu$ l of each glycine concentration was pipetted into a well, the different concentrations used in this study were 0 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 10 mM, 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 120 mM, 150 mM, 180 mM and 200 mM. The total number of reactions for all of the different benzoyl-CoA and glycine concentrations were 1400 reactions.

The master mix, benzoyl-CoA and glycine was incubated at 37°C whilst the final 96-well plate was prepared. A negative control was made by eliminating glycine. While the substrates incubated, the plate reader was set up according to the following specifications: the temperature at which the reactions were carried out was 37°C, absorbance measured at 412nm every 41 seconds for 20 minutes. In a clean 96-well plate, 2 $\mu$ g of the purified GLYAT enzyme was added. The next three steps were carried out rapidly.

20  $\mu$ l of the benzoyl-CoA was added to the 96-well plate, already containing the enzyme (2 $\mu$ g), 40  $\mu$ l of each glycine concentration was added followed by the master mix with a final volume of the reaction of 200 $\mu$ l (see Table 4.3).

The plate was placed into the plate reader (BioTek). The Gen5 software programme was used with the plate reader for data interpretation.

**Table 4.3:** Example of the reaction composition in each 96-well plate.

<b>Reaction per well:</b>	0.01mM DTNB (diluted in Tris-Cl)
	2 µg enzyme
	40 µL glycine (100mM)
	20 µL benzoyl-CoA (150µM)
	Nuclease-free water to a final volume of 200µL
<b>Final volume:</b>	200µL

#### 4.5 Data processing to determine the GLYAT kinetic parameters

The data obtained from the bi-substrate kinetic analyses was absorbance ( $A_{412}$ ; x-axis) over time (minutes; y-axis). Linear regression was used on each individual plot (e.g. 100µM Benzoyl-CoA; 150mM glycine) to find the linear portion of the graph in order to determine the initial velocity ( $V_0$ ) of each plot. The initial velocity values (in triplicate) for each substrate combination were used to test different enzyme kinetic models (Michaelis-Menten; sigmoidal) to determine the model with the best fit ( $X^2$  value). The kinetic parameters determined by the best fit model was converted from absorbance/min to nmol/min/mg. In order to do this a conversion factor was calculated, see appendix for the conversion calculations.

#### 4.6 Determining substrate specificity

The substrate specificity was determined to see if the different haplotype variants have an effect on substrate specificity of GLYAT. Because the kinetic model is a sigmoidal mechanism the normal way to determine substrate specificity changed (Ebrecht *et al.*,2017). For sigmoidal enzymes the substrate specificity is determined by calculation of the catalytic turnover number,  $K_{cat}$  ( $s^{-1}$ ) divided by the substrate concentration,  $S_{0.5}$ (mM). Substrate specificity is measured in  $s^{-1}mM^{-1}$ . [Et] used for  $K_{cat}$  is the total enzyme concentration, the protein concentration used was 2µg.

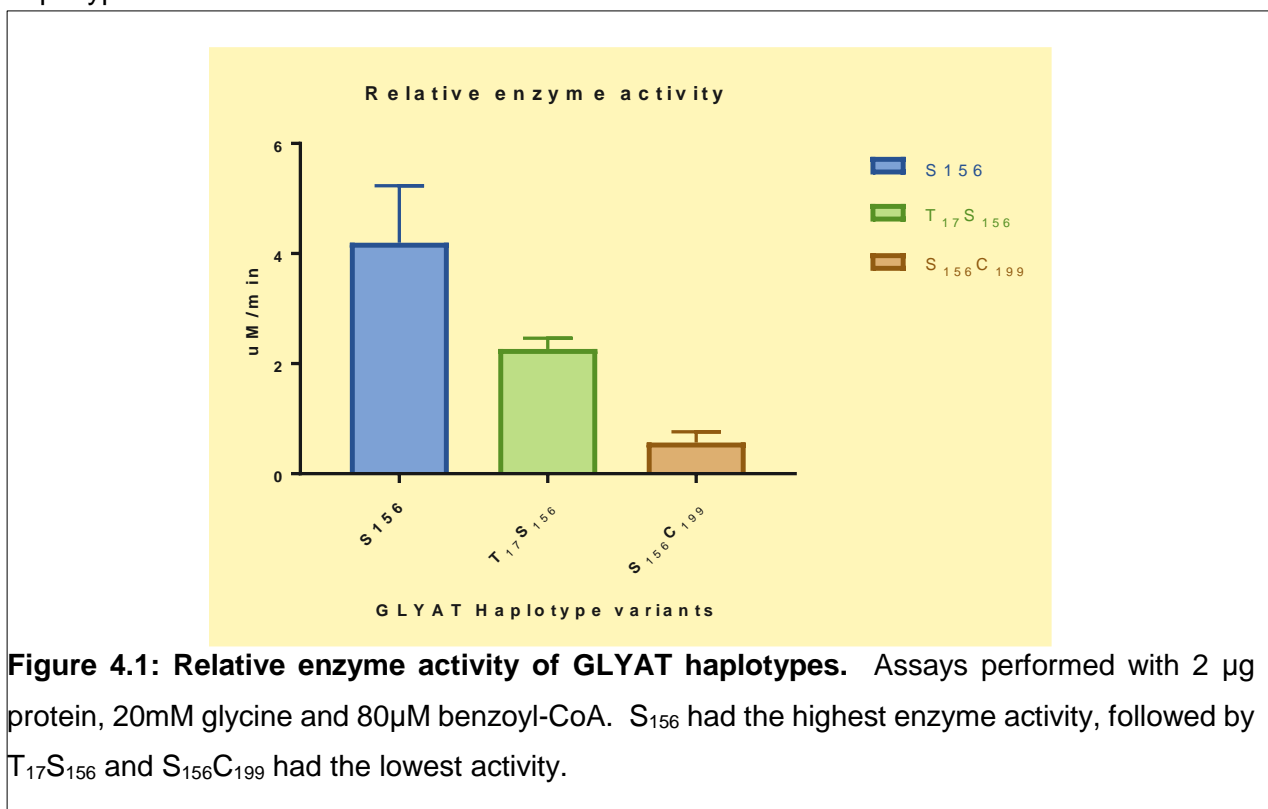
$$K_{\text{cat}} = \frac{V_{\text{max}}}{[E]_t}$$

$$\text{Substrate specificity} = \frac{K_{\text{cat}} (s^{-1})}{S_{0.5} (mM)}$$

## 4.7 Results and Discussion

### 4.7.1 Relative enzyme activity

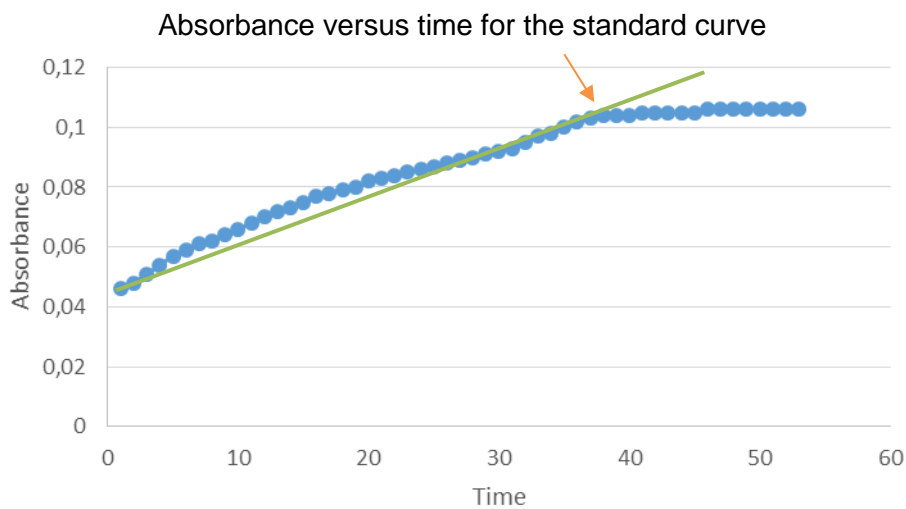
After successful purification of the three variants (section 3.5.8), the relative enzyme activity of each variant was determined. From Figure 4.1, it can be seen that S<sub>156</sub> had the highest relative enzyme activity when compared to the other two variants (4.19µM/min). T<sub>17</sub>S<sub>156</sub> had the second highest activity (2.6µM/min) followed by S<sub>156</sub>C<sub>199</sub> with the lowest activity (1.7µM/min). Van der Sluis *et al.* (2013) determined the relative enzyme activities for three enzymes, S<sub>156</sub>, S<sub>17</sub>T and R<sub>199</sub>C. The S<sub>156</sub> had the highest enzyme activity (970 nmol/min/mg) of all the SNP's tested, the S<sub>17</sub>T had the third highest relative enzyme activity (700nmol/min/mg) and R<sub>199</sub>C had almost no activity, making this SNP vertically inactive. This compares with the finding for the different haplotypes.



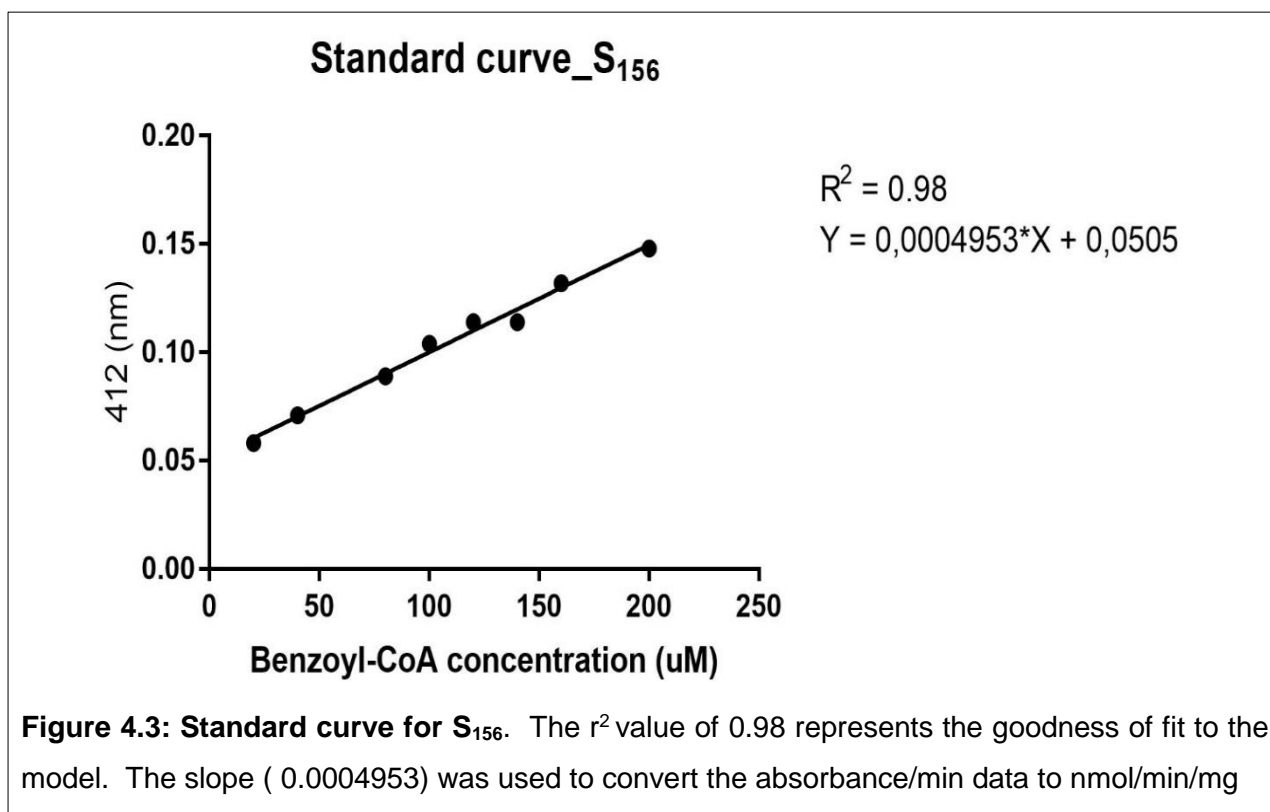
### 4.7.2 Standard curve of the S<sub>156</sub> variant

A standard curve characterises the relationship between two measures that are used to determine values or concentrations for substances. The standard curve was done to determine the value of the slope that was used for data processing. The end-point assay for the standard curve is explained in section 4.3. The standard curve reactions were allowed to completely finish (benzoyl-CoA and glycine conjugated to hippurate). Figure 4.2 is as a representation of such a

graph. When the reactions were completed, there was no more CoASH released to react with DTNB, thus causing a plateau to form. A linear regression was used to determine the linear part of the graph before the reaction reached its plateau (represented by the green line on Figure 4.2). Each data point on the standard curve is an average of the triplicates of each end-point. Figure 4.3 shows the data used (this is an average of the three endpoint reactions) when the reaction reached its plateau, a linear increase with concentration and a  $r^2$  value can be seen as a result. The slope 0,0004953 was further used in the kinetic data processes (Section 4.5).



**Figure 4.2:** Representation of the graph (absorbance versus time) used for the standard curve. The data used to compile the standard curve is where the graph reached its plateau. The orange arrow represents the data point that was used for the construction for the standard curve



#### 4.7.3 Bi-substrate kinetics

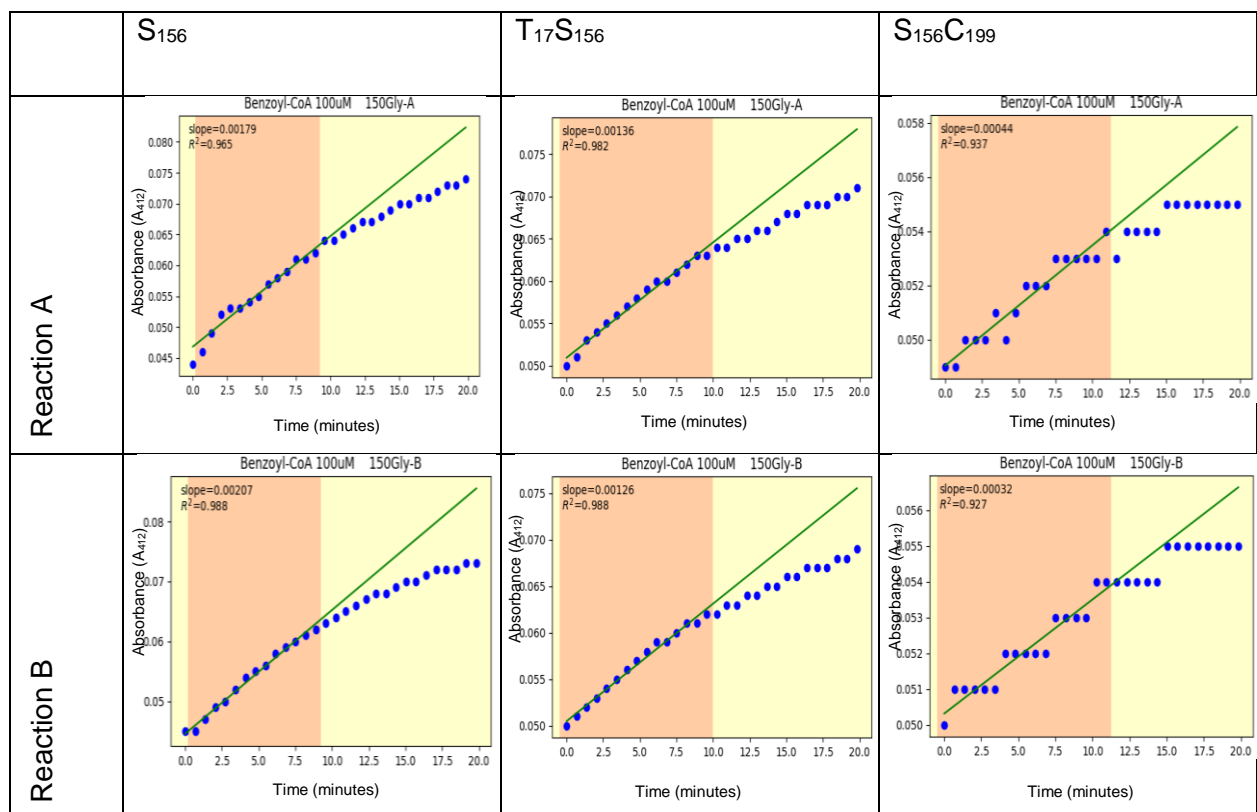
To determine the bi-substrate kinetic parameters for S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub> both substrates (benzoyl-CoA and glycine) concentrations were varied. The benzoyl-CoA concentrations ranged from 0 – 200µM while glycine was kept constant and the glycine concentrations were varied from 0 – 200mM while benzoyl-CoA was kept constant. In Table 4.4 – 4.6, the raw data for the bi-substrate kinetics for the three variants are shown; the data was obtained with benzoyl-CoA concentration of 100µM and glycine concentration of 150mM. This is just the data for one reaction in triplicate. To avoid repetition the remaining raw data is given in the appendix for all three proteins with all their different benzoyl-CoA and glycine concentrations. Table 4.4 – 4.6 shows the protein of interest, the three reactions, the standard deviation and the %CV (Coefficient of variation) value.

The CV value is usually represented in %; it indicates the variation of the data around the mean. If the %CV is high, it indicates that the variation of data is large and a lower %CV value means a low level of variation of data. The data referred to for the %CV values are the data from the triplicates. The lower the %CV the more precise the data. Any data is acceptable if it falls within the range of 0 to 15% (Whitmire *et al.*, 2011). In all three the tables and appendix, it can be seen that the triplicates are almost all the same with a slight variation regarding standard deviation and low %CV values. The %CV values for the three variants are between 0 – 3.46% with only one

10% CV value. See appendix for Tables 4.4 - 4.6 that shows the protein of interest, the three reactions, the standard deviation and the %CV (Coefficient of variation) value

The data obtained (see Table 4.4 - 4.6) was used to draw graphs of the results. The time was on x-axis and the absorbance values on the y-axis. The reactions were done in triplicate for each of the proteins. Linear regression was used on each individual plot (e.g. 100µM Benzoyl-CoA; 150mM glycine) to find the linear portion of the graph in order to determine the initial velocity ( $V_0$ ) of each plot. Table 4.7 shows the graphs for the data obtained during this study, this is however just an extract of all the graphs. As can be seen from Table 4.4 – 4.7 the area shaded in orange was used to determine the initial velocity (Abs/min). The initial velocity values (in triplicate) for each substrate combination were used to test different enzyme kinetic models (Michaelis-Menten; sigmoidal) to determine the model with the best fit ( $\chi^2$  value).

**Table 4.7:** Absorbance vs time graphs of the three proteins with benzoyl-CoA concentration 100µM and glycine 150mM. Reaction A, B and C are the reactions performed in triplicate.



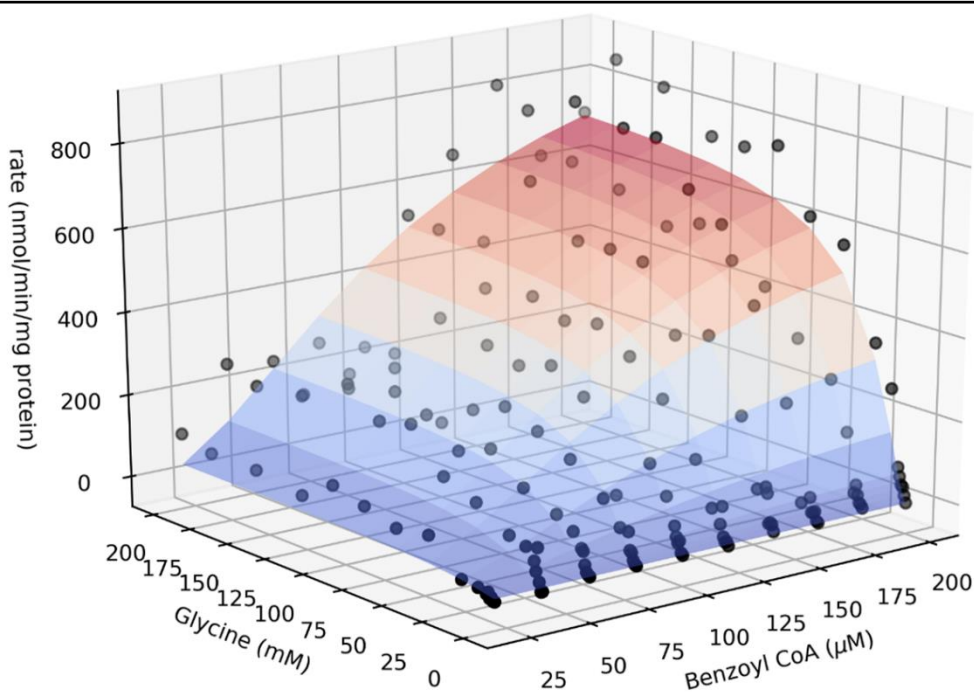
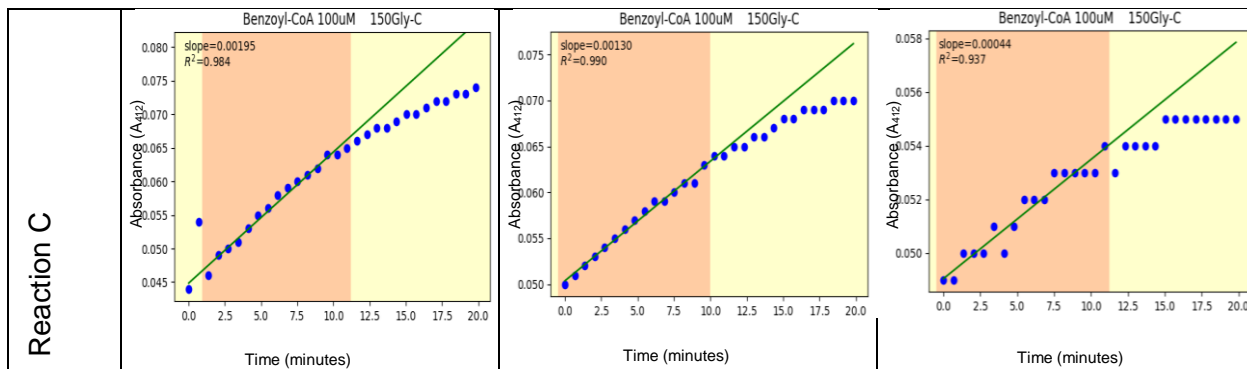
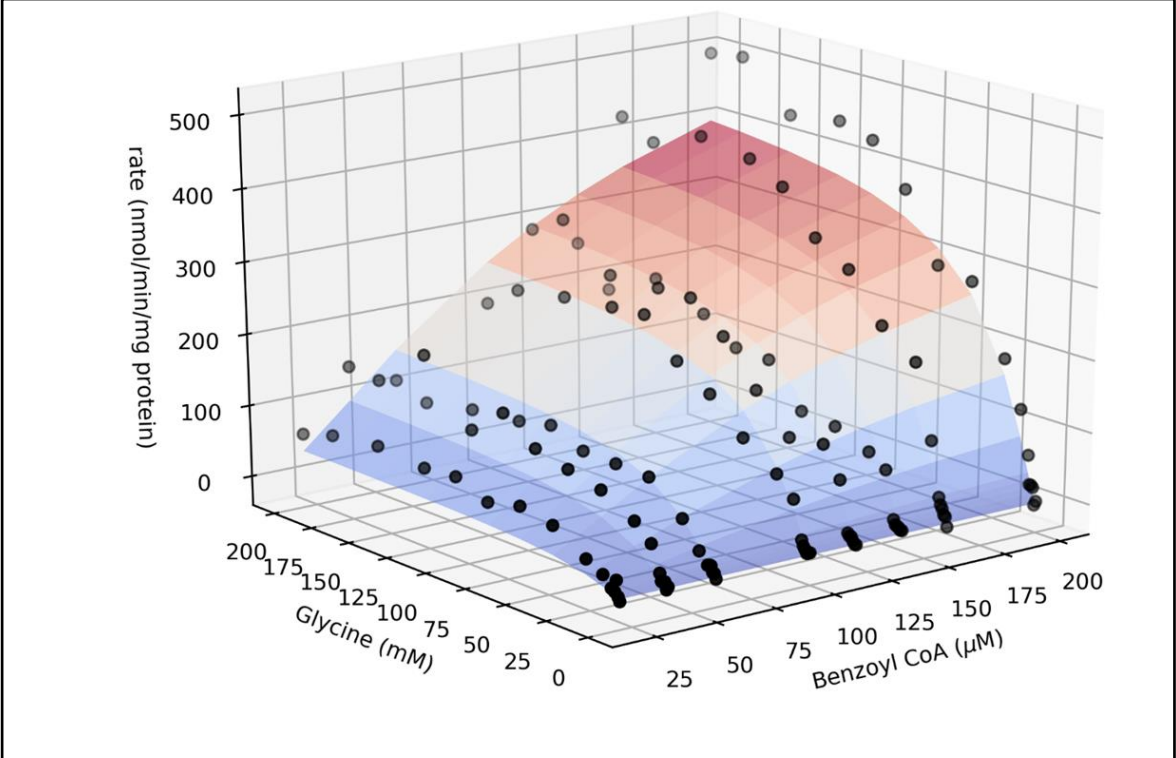
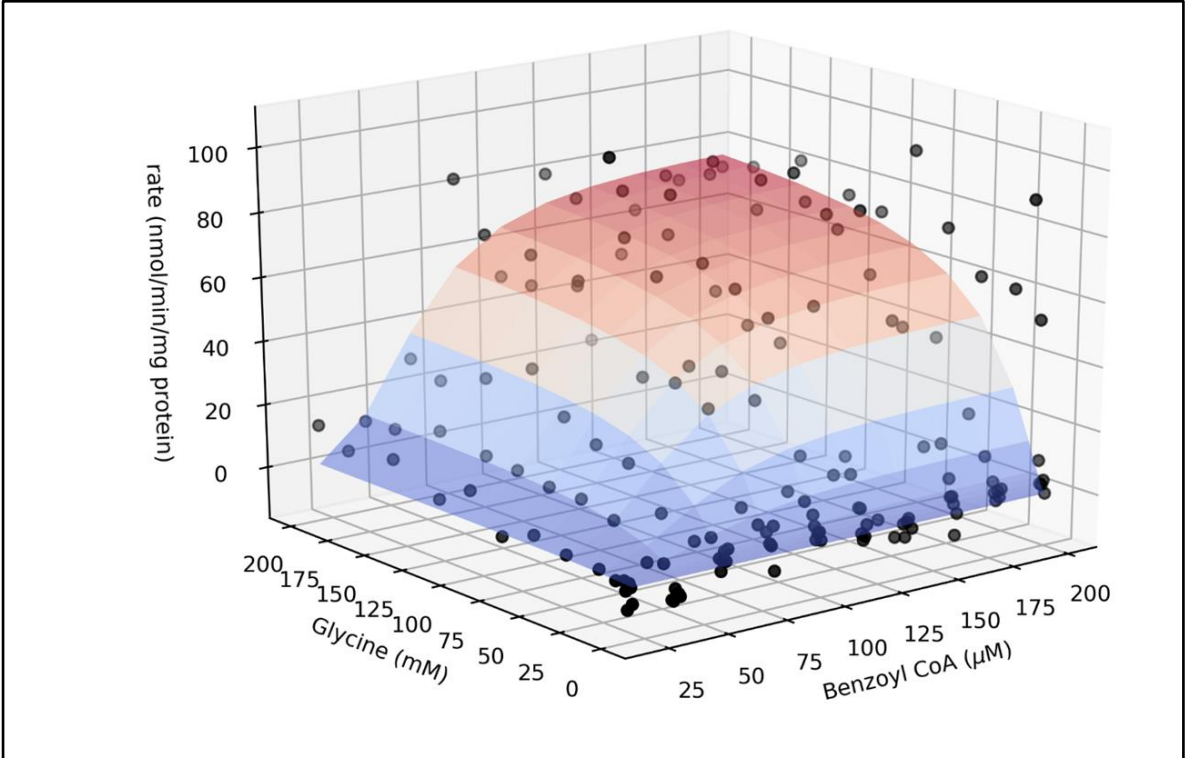


Figure 4.4: Representation of a 3D graph of the S<sub>156</sub> variant



**Figure 4.5:** Representation of a 3D graph of the T<sub>17</sub>S<sub>156</sub> variant



**Figure 4.6:** Representation of a 3D graph of the S<sub>156</sub>C<sub>199</sub> variant

#### 4.7.4 Kinetic model for each of the GLYAT variants

Table 4.8 shows the results obtained for the bi-substrate kinetic analysis of the three variants after a global fit of the data was performed using the sigmoidal enzyme kinetic model. The  $\chi^2$  value is an indication to show the quality of the fit of the model, the smaller the number the better the data fits the model. All three variants have a very small number, indicating a good fit of the model. The  $K_{gly}$  (glycine) and  $K_{benz}$  (benzoyl-CoA) is the half saturating constants, they represent the concentration of substrate that will give half of  $V_{max}$  if the other substrate is in abundance. The  $K_{gly}$  for the  $S_{156}$  ( $23.9 \pm 1.8\text{mM}$ ) and the  $S_{156}C_{199}$  ( $24.2 \pm 12.0\text{mM}$ ) variants were lower when compared to the  $K_{gly}$  values of  $T_{17}S_{156}$  ( $28.7 \pm 3.1\text{mM}$ ), which is an indication that the  $S_{156}$  and the  $S_{156}C_{199}$  have a higher affinity for glycine. The  $K_{benz}$  value for  $S_{156}C_{199}$  is the lowest of all the variants, this is an indication that this variant has the highest affinity for benzoyl-CoA compared to the others, this might explain the slow turnover and low enzyme activity of this variant as the substrate remains tightly bound.

The Hill equation was used to fit the data and the software used was Python. Equation 4.1 represents the Hill equation.  $V$  is the reaction velocity in a unit of time,  $V_{max}$  is the maximum velocity of the reaction,  $[S]$  is the substrate concentration and  $K_{0.5}$  is half the  $V_{max}$ ,  $h$  is the Hill coefficient.

$$v = \frac{V_{max} \times [S]^h}{(K_{0.5})^h + [S]^h}$$

The Hill coefficient ( $h$ ) represents the affinity of the enzyme for the substrate. More than one binding site on an enzyme can lead to the possibility of interaction between the binding sites during the process of binding and is termed cooperativity. Cooperativity is reflected by the value of  $h$ . Positive cooperativity ( $h > 1$ ) is the binding of a substrate resulting in the increase of the affinity for the remaining substrates, the same or different, to bind to the enzyme. Negative cooperativity ( $h < 1$ ) occurs as the binding of one substrate to the protein will decrease the affinity for the other substrates, the same or different, to bind. Non-cooperativity ( $h = 1$ ) is independent substrate binding, the substrates will bind to the enzyme regardless of one another, this is normal Michaelis-Menten (Palmer & Bonner, 2007; PhysiologyWeb, 2013).

The  $h_{gly}$  (glycine) and  $h_{benz}$  (benzoyl-CoA) refers to the Hill coefficient. The  $h_{gly}$  for  $S_{156}C_{199}$  is smaller than 1, this means that the binding of glycine to GLYAT will decrease the affinity for benzoyl-CoA. The other two variants had  $h_{gly}$  and  $h_{benz} > 1$ , this means positive cooperativity: both the binding of glycine or benzoyl-CoA increases the affinity for the other substrate.

**Table 4.8:** Different kinetic parameters for the three GLYAT variants

Recombinant variant	$\chi^2 (e^{-0.5})$	Vf (nmol/min/mg)	K <sub>gly</sub> (mM)	h <sub>gly</sub>	k <sub>benz</sub> (mM)	h <sub>benz</sub>
<b>S<sub>156</sub></b>	8.47	951 ± 50.5	23.9 ± 1.8	1.47 ± 0.12	106.7 ± 3.6	1.94 ± 0.10
<b>T<sub>17</sub>S<sub>156</sub></b>	2.12	632 ± 23	28.7 ± 3.1	1.31 ± 0.12	118.0 ± 7.2	1.49 ± 0.08
<b>S<sub>156</sub>C<sub>199</sub></b>	8.60	80.75 ± 6.1	24.2 ± 12.0	0.88 ± 0.30	60.3 ± 10.8	5.34 ± 4.76

#### 4.7.5 Substrate specificity

Substrate specificity for sigmoidal enzymes are determined using the catalytic turnover number ( $K_{cat}$ ) divided by the substrate concentration that will give half of the  $V_{max}$  value ( $S_{0.5}$ ). The substrate specificity for all three variants were determined. Table 4.9 shows the results for the variants. For sigmoidal enzymes the higher the  $K_{cat}/S_{0.5}$  value the more specificity the enzyme has towards the substrate (Ebrecht *et al.*,2017).  $S_{156}$  had the highest substrate specificity for glycine of  $19.89 \text{ s}^{-1}\text{mM}^{-1}$  and for benzoyl-CoA of  $4.46 \text{ s}^{-1}\text{mM}^{-1}$ ,  $T_{17}S_{156}$  had the second highest substrate specificity for both glycine and benzoyl-CoA with  $11.01 \text{ s}^{-1}\text{mM}^{-1}$  and  $2.68 \text{ s}^{-1}\text{mM}^{-1}$  respectively.  $S_{156}C_{199}$  had the lowest substrate specificity values with  $1.65 \text{ s}^{-1}\text{mM}^{-1}$  for glycine and  $0.66 \text{ s}^{-1}\text{mM}^{-1}$  for benzoyl-CoA.

**Table 4.9:** The different substrate specificity for the three GLYAT variants.

Haplotype variant	Substrate specificity for glycine $K_{cat}/S_{0.5} (\text{s}^{-1}\text{mM}^{-1})$	Substrate specificity or Benzoyl-CoA $K_{cat}/S_{0.5} (\text{s}^{-1}\text{mM}^{-1})$
<b>S<sub>156</sub></b>	19.89	4.46
<b>T<sub>17</sub>S<sub>156</sub></b>	11.01	2.68
<b>S<sub>156</sub>C<sub>199</sub></b>	1.65	0.66

## 4.8 Summary

The three GLYAT haplotype enzymes were used in kinetic studies to determine the relative enzyme kinetics, the bi-substrate kinetics and the substrate specificity. The S<sub>156</sub> variant showed the highest enzyme activity, the T<sub>17</sub>S<sub>156</sub> had the second highest activity, around half of the S<sub>156</sub> variant. The S<sub>156</sub>C<sub>199</sub> variant had the lowest activity, more or less half of the T<sub>17</sub>S<sub>156</sub> variant and barely comparable with the wild-type variant. These findings correspond with van der Sluis *et al.*, (2013) where they used the SNPs to determine the relative enzyme activities (van der Sluis *et al.*, 2013). S<sub>156</sub> haplotype had the highest relative enzyme activity when compared to the other two variants (4,19μM). T<sub>17</sub>S<sub>156</sub> had the second highest activity (2,6μM) followed by S<sub>156</sub>C<sub>199</sub> with the lowest activity (1,7μM). Van der Sluis *et al.* (2013) determined the relative enzyme activities for three SNP enzymes, S<sub>156</sub>, S<sub>17</sub>T and R<sub>199</sub>C. The S<sub>156</sub> had the highest enzyme activity (970 nmol/min/mg) of all the SNP's tested, the S<sub>17</sub>T had the third highest relative enzyme activity (700nmol/min/mg) and R<sub>199</sub>C had almost no activity, making this SNP virtually inactive.

The bi-substrate kinetic characterisation was done with varying benzoyl-CoA concentrations of 0μM - 200μM while glycine was kept constant and glycine concentrations of 0mM – 200mM.

The model that GLYAT follows according to the data is the Hill model, which displays cooperativity. The Hill coefficient for the S<sub>156</sub> and T<sub>17</sub>S<sub>156</sub> variants for Benzoyl-CoA and glycine are larger than one, which means positive cooperativity. The Hill coefficient for glycine resulted in negative cooperativity for the S<sub>156</sub>C<sub>199</sub> variant, thus decreasing the affinity for benzoyl-CoA to bind to the enzyme.

The substrate specificity was determined for all three variants, the S<sub>156</sub> variant had the highest substrate specificity for both glycine and benzoyl-CoA, T<sub>17</sub>S<sub>156</sub> had the second highest substrate specificity and the S<sub>156</sub>C<sub>199</sub> variant had the lowest substrate specificity.

## CHAPTER 5: CONCLUSION AND FUTURE PROSPECTS

### 5.1 Introduction

The glycine conjugation pathway is proposed to be a vital pathway in the human body (van der Sluis *et al.*, 2015). This pathway is mainly responsible for the conjugation of benzoyl-CoA and glycine to form hippurate. There are more substrates that this pathway will detoxify such as salicyl-CoA, isovaleryl-CoA, propionyl-CoA and octanoyl-CoA. Since so much additives and preservatives are added the amount of ingested benzoic acid consumed daily by an individual has increased immensely (del Olmo *et al.*, 2017). This in turn puts a lot of strain on the glycine conjugation pathway. The GLYAT enzyme is responsible for the conjugation of the amino acid and the acyl-CoA to form the product that can be excreted. It is necessary to characterise this enzyme to see what the influence of the different haplotypes are on the enzyme and because this could influence the whole pathway.

GLYAT is the enzyme responsible for the conjugation of benzoyl-CoA with glycine and releases the by-product CoASH. There are genetic variations in the *GLYAT* gene that can cause variation in the GLYAT enzyme to form different haplotypes (van der Sluis *et al.*, 2015). In this study only three haplotypes of the GLYAT enzyme were evaluated, the S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and the S<sub>156</sub>C<sub>199</sub>. These three haplotypes were chosen because the SNPs were tested and van der Sluis *et al* found that the S<sub>156</sub> variant is the wild-type variant with the highest enzyme activity. S<sub>17</sub>T had the second highest enzyme activity and then R<sub>199</sub>C had almost no enzyme activity (van der Sluis *et al.*, 2015). The bi-substrate kinetics and substrate specificity for these enzymes were determined followed by a new kinetic model that will help to better describe the activity of the GLYAT enzyme.

### 5.2 Conclusion

#### 5.2.1 Expression and purification of S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub>

In conclusion, all of the objectives were successfully reached. For objective one the T<sub>17</sub>S<sub>156</sub> variant was constructed using the Phusion High-Fidelity DNA Polymerase. The expression of S<sub>156</sub>, T<sub>17</sub>S<sub>156</sub> and S<sub>156</sub>C<sub>199</sub> in a soluble fraction was successfully achieved using the pET expression system in Origami cells expressing the GroEL and GroES chaperones as described in Section 3.4. GLYAT and GroEL (~52kDa) both express at the same size, purification was needed to verify that it was indeed GLYAT being expressed and not just GroEL and, as enzyme kinetics characterisation was performed on the GLYAT enzymes purified proteins were needed. The three proteins were subsequently purified using His-tags and nickel-affinity columns as all three proteins were expressed with 6 x His tags.

### 5.2.2 Enzyme kinetics for the three different GLYAT variants

The second objective of the study was to compare the relative enzyme activity of the three different haplotypes. The enzyme activity was tested using the method described in Section 4.2. A colometric assay was used which detects the colour change between the released CoASH and the DNTB. This is not the most sensitive method but accurate enough to test for the production of hippurate.

Different relative enzyme activities are available for some SNPs of GLYAT, but no haplotypes have been characterised before (van der Sluis *et al.*, 2013). We wanted to characterise haplotypes because haplotypes are a gene set (genetic markers) that is arranged close to each other on a chromosome and is also inherited as one biological unit, haplotypes are also not easily separated by recombination (Ciurea *et al.*, 2019). There are different known haplotype variants that can add to our understanding of exactly how these variants influences the GLYAT enzyme activity. S<sub>156</sub> had the highest enzyme activity (wild-type), T<sub>17</sub>S<sub>156</sub> had the second highest activity and S<sub>156</sub>C<sub>199</sub> had the lowest activity.

### 5.2.3 The bi-substrate kinetics for the three variants

Most of the literature assumed that human GLYAT will also follow the sequential Bi-Bi mechanism like bovine GLYAT (Kelley & Vessey, 1994; Mawal & Qureshi, 1994; van der Westhuizen *et al.*, 2000; Matsuo *et al.*, 2012; van der Sluis *et al.*, 2013). A preliminary study on the bi-substrate kinetics for the recombinant human GLYAT (S<sub>156</sub>) indicated a sigmoidal enzyme model when glycine concentrations were smaller or equal to 5mM. Glycine concentrations higher than 5mM gave the normal Michealis-Menten mechanism (van der Sluis *et al.*, 2017).

There are sulfotransferase enzymes (SULT) that when their enzyme kinetic mechanism were studied over a small concentration range showed Michaelis-Menten kinetics. As the concentration range became wider, cooperativity was exhibited (James MO., 2014).

The third objective was to use all three variants and determine the bi-substrate kinetics. The benzoyl-CoA concentration was varied from 0 - 200µM with a constant glycine concentration and secondly the glycine concentration was varied from 0 – 200 mM while benzoyl-CoA was kept constant. A global fit of the data was performed using sigmoidal enzyme kinetics, this correlates with the previous study done (van der Sluis *et al.*, 2017). These finding aid in the big literature variation of the K<sub>m</sub> values for GLYAT as the K<sub>0.5</sub> values differ for sigmoidal enzymes with different substrate concentrations because sigmoidal enzymes are cooperative enzymes, having an ideal substrate concentration that will result in V<sub>max</sub>. The K<sub>0.5</sub> value (the value of half of V<sub>max</sub>) will thus change as the substrate concentration changes (Palmer & Bonner, 2007; Bhagavan and Ha,

2015). This enzyme is more complex than originally thought and needs to be further characterised.

### 5.3 Future prospects

Because the glycine conjugation pathway can conjugate different acyl-CoAs, isovaleryl-CoA is one of the substrates that will conjugate with glycine to form isovalerylglycine (see Section 2.8.2 on organic acidemias). This can then be excreted in the urine. If benzoyl-CoA and salicyl-CoA is not in the system the next substrate of preference for GLYAT is isovaleryl-CoA (see Table 2.3 on substrate specificity).

We attempted to do the bi-substrate for isovaleryl-CoA using the S<sub>156</sub> protein with glycine concentration kept constant at 200 mM and isovaleryl-CoA concentration was 40µM, 140µM and 200µM. The colometric method was not sensitive enough for useful data. A new method will be developed using the Mass Spectrometer in future studies.

A method needs to be optimised on the Mass Spectrometer that would be able to monitor the substrate disappearance against product formation over a certain time, which can be done for the substrates for which GLYAT has a low affinity. The limitations are the high concentration of Tris (25mM) that the reaction needs, which is not compatible with the MS (Xia, DeGrandchamp and Williams., 2019). Future studies can be done on a concentration gradient for benzoyl-CoA and glycine.

Only three variants were used in this study, a study needs to be done on all the other GLYAT haplotypes to determine the different relative enzyme activities and bi-substrate kinetics. This will help us understand if people have these variants how this will influence their glycine conjugation pathway.

A study can be done to develop a knock out mouse model for GLYAT to test the importance of the glycine conjugation pathway as it is seen as an essential pathway (van der Sluis *et al.*, 2015).

A co-expression model with both the two enzymes (ACSM2 and GLYAT) can be made to test different affinities for the substrate as well as how the variations of the enzymes will influence detoxification of the glycine conjugation pathway. Benzoyl-CoA is the preferred substrate for GLYAT followed by salicylate. If benzoate and Salicylate are administered at the same time the benzoate will be conjugated to glycine first followed by the salicylate. A co-expression model will help with classifying the substrate specificity for both enzymes on the different acyl-CoAs.

Organic acidemias is a disease where the body has a faulty enzyme and the inability to excrete certain acyl-CoA's. Some of these acyl-CoA's are also substrates for the glycine conjugation pathway. The glycine conjugation pathway can conjugate glycine and the different acyl-CoA's (e.g. isovaleryl-CoA and propionyl-CoA) to form a product than can easily be excreted (Tanaka & Isselbacher, 1967). Further studies can be done with different substrates (those that are responsible for organic acidemias) to determine how the glycine conjugation pathway can aid patients with organic acidemias.

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## ANNEXURES

**Table 4.2:** An example of a 96-well plate layout with varying glycine concentrations (mM) and benzoyl-CoA (20 $\mu$ M) used to determine bi-substrate kinetics.

		1	2	3	4	5	6
A	Glycine	1	1	1	60	60	60
	Benzoyl-CoA	20	20	20	20	20	20
B	Glycine	2	2	2	80	80	80
	Benzoyl-CoA	20	20	20	20	20	20
C	Glycine	3	3	3	100	100	100
	Benzoyl-CoA	20	20	20	20	20	20
D	Glycine	4	4	4	120	120	120
	Benzoyl-CoA	20	20	20	20	20	20
E	Glycine	5	5	5	150	150	150
	Benzoyl-CoA	20	20	20	20	20	20
F	Glycine	10	10	10	180	180	180
	Benzoyl-CoA	20	20	20	20	20	20
G	Glycine	20	20	20	200	200	200
	Benzoyl-CoA	20	20	20	20	20	20
H	Glycine	40	40	40			
	Benzoyl-CoA	20	20	20			
I	Glycine	0	0	0			

	Benzoyl-CoA	20	20	20			
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**Conversion from absorbance/min to nmol/min/mg:**

STD curve equation  $y=0.0004953X+0.0505$

Slope =  $0.0004953 \mu\text{M}^{-1}$

$$c = (v_0/\text{min}) / (0.0004953/\mu\text{M})$$

$$= v_0/\text{min} \times \mu\text{M}/0.0004953$$

$$= (v_0/0.0004953) \mu\text{M}/\text{min}$$

$$= (v_0/0.0004953) (\text{micromole}/\text{L})/\text{min}$$

$$= (v_0/0.0004953) (\text{nanomole}/\text{ml})/\text{min}$$

$$= [(v_0/0.0004953) (\text{nanomole}/\text{ml})/\text{min}] \times 0.2 \text{ ml (reaction volume} \rightarrow 200 \text{ ul)}$$

$$= (0.2 v_0/0.0004953) \text{ nanomole}/\text{min}$$

$$= 403,7957 v_0 \text{ nanomole}/\text{min for 2 ug of protein}$$

$$= (403,7957 v_0 \text{ nanomole}/\text{min}) / 2 \text{ ug}$$

$$= (201,8978 v_0 \text{ nanomole}/\text{min}) / 1 \text{ ug}$$

$$= (201,8978 v_0 \text{ nanomole}/\text{min}) / 0.001 \text{ mg}$$

$$= 201\,897,8 v_0 \text{ nanomole}/\text{min}/\text{mg}$$

E.g.  $S_{156}$  reaction

$$V_f = 0.00471$$

Therefore:  $201\,897,8 (0.00471) \text{ nanomole}/\text{min}/\text{mg}$

$$= 950,94 \text{ nmole}/\text{min}/\text{mg}$$

= 0.951 umol/min/mg

**Table 4.4:** Bi-substrate enzyme kinetics of S<sub>156</sub> with benzoyl-CoA 100µM and glycine 150mM

<b>S<sub>156</sub></b>	<b>Time (minutes)</b>	<b>Reaction A</b>	<b>Reaction B</b>	<b>Reaction C</b>	<b>Standard deviation</b>	<b>%CV</b>
	00:00	0,044	0,045	0,044	0,000577	1,30
	00:41	0,046	0,045	0,054	0,004933	10,21
	01:22	0,049	0,047	0,046	0,001528	3,23
	02:03	0,052	0,049	0,049	0,001732	3,46
	02:44	0,053	0,05	0,05	0,001732	3,40
	03:25	0,053	0,052	0,051	0,001	1,92
	04:06	0,054	0,054	0,053	0,000577	1,08
	04:47	0,055	0,055	0,055	0	0
	05:28	0,057	0,056	0,056	0,000577	1,02
	06:09	0,058	0,058	0,058	0	0
	06:50	0,059	0,059	0,059	0	0
	07:31	0,061	0,06	0,06	0,000577	0,96
	08:12	0,061	0,061	0,061	0	0
	08:53	0,062	0,062	0,062	0	0
	09:34	0,064	0,063	0,064	0,000577	0,91
	10:15	0,064	0,064	0,064	0	0
	10:56	0,065	0,065	0,065	0	0
	11:37	0,066	0,066	0,066	0	0

	12:18	0,067	0,067	0,067	0	0
	12:59	0,067	0,068	0,068	0,000577	0,85
	13:40	0,068	0,068	0,068	0	0
	14:21	0,069	0,069	0,069	0	0
	15:02	0,07	0,07	0,07	0	0
	15:43	0,07	0,07	0,07	0	0
	16:24	0,071	0,071	0,071	0	0
	17:05	0,071	0,072	0,072	0,000577	0,81
	17:46	0,072	0,072	0,072	0	0
	18:27	0,073	0,072	0,073	0,000577	0,79
	19:08	0,073	0,073	0,073	0	0
	19:49	0,074	0,073	0,074	0,000577	0,78

**Table 4.5:** Bi-substrate kinetics of T<sub>17</sub>S<sub>156</sub> with benzoyl-CoA 100μM and glycine 150mM

<b>T<sub>17</sub>S<sub>156</sub></b>	<b>Time (minutes)</b>	<b>Reaction A</b>	<b>Reaction B</b>	<b>Reaction C</b>	<b>Standard deviation</b>	<b>%CV</b>
	00:00	0,05	0,05	0,05	8,5E-18	1,70E-14
	00:41	0,051	0,051	0,051	0	0
	01:22	0,053	0,052	0,052	0,000577	1,10
	02:03	0,054	0,053	0,053	0,000577	1,08
	02:44	0,055	0,054	0,054	0,000577	1,06
	03:25	0,056	0,055	0,055	0,000577	1,04
	04:06	0,057	0,056	0,056	0,000577	1,02

	04:47	0,058	0,057	0,057	0,000577	1,01
	05:28	0,059	0,058	0,058	0,000577	0,99
	06:09	0,06	0,059	0,059	0,000577	0,97
	06:50	0,06	0,059	0,059	0,000577	0,97
	07:31	0,061	0,06	0,06	0,000577	0,96
	08:12	0,062	0,061	0,061	0,000577	0,94
	08:53	0,063	0,061	0,061	0,001155	1,87
	09:34	0,063	0,062	0,063	0,000577	0,92
	10:15	0,064	0,062	0,064	0,001155	1,82
	10:56	0,064	0,063	0,064	0,000577	0,91
	11:37	0,065	0,063	0,065	0,001155	1,79
	12:18	0,065	0,064	0,065	0,000577	0,89
	12:59	0,066	0,064	0,066	0,001155	1,77
	13:40	0,066	0,065	0,066	0,000577	0,88
	14:21	0,067	0,065	0,067	0,001155	1,74
	15:02	0,068	0,066	0,068	0,001155	1,79
	15:43	0,068	0,066	0,068	0,001155	1,71
	16:24	0,069	0,067	0,069	0,001155	1,69
	17:05	0,069	0,067	0,069	0,001155	1,69
	17:46	0,069	0,067	0,069	0,001155	1,69
	18:27	0,07	0,068	0,07	0,001155	1,67
	19:08	0,07	0,068	0,07	0,001155	1,67

	19:49	0,071	0,069	0,07	0,001	1,43
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**Table 4.6:** Bi-substrate kinetics of S<sub>156</sub>C<sub>199</sub> with benzoyl-CoA 100μM and glycine 150mM

<b>S<sub>156</sub>C<sub>199</sub></b>	<b>Time (minutes)</b>	<b>Reaction A</b>	<b>Reaction B</b>	<b>Reaction C</b>	<b>Standard deviation</b>	<b>%CV</b>
	00:00	0,049	0,05	0,049	0,000577	1,17
	00:41	0,049	0,051	0,049	0,001155	2,32
	01:22	0,05	0,051	0,05	0,000577	1,15
	02:03	0,05	0,051	0,05	0,000577	1,15
	02:44	0,05	0,051	0,05	0,000577	1,15
	03:25	0,051	0,051	0,051	0	0
	04:06	0,05	0,052	0,05	0,001155	2,28
	04:47	0,051	0,052	0,051	0,000577	1,12
	05:28	0,052	0,052	0,052	0	0
	06:09	0,052	0,052	0,052	0	0
	06:50	0,052	0,052	0,052	0	0
	07:31	0,053	0,053	0,053	0	0
	08:12	0,053	0,053	0,053	0	0
	08:53	0,053	0,053	0,053	0	0
	09:34	0,053	0,053	0,053	0	0
	10:15	0,053	0,054	0,053	0,000577	1,08
	10:56	0,054	0,054	0,054	0	0

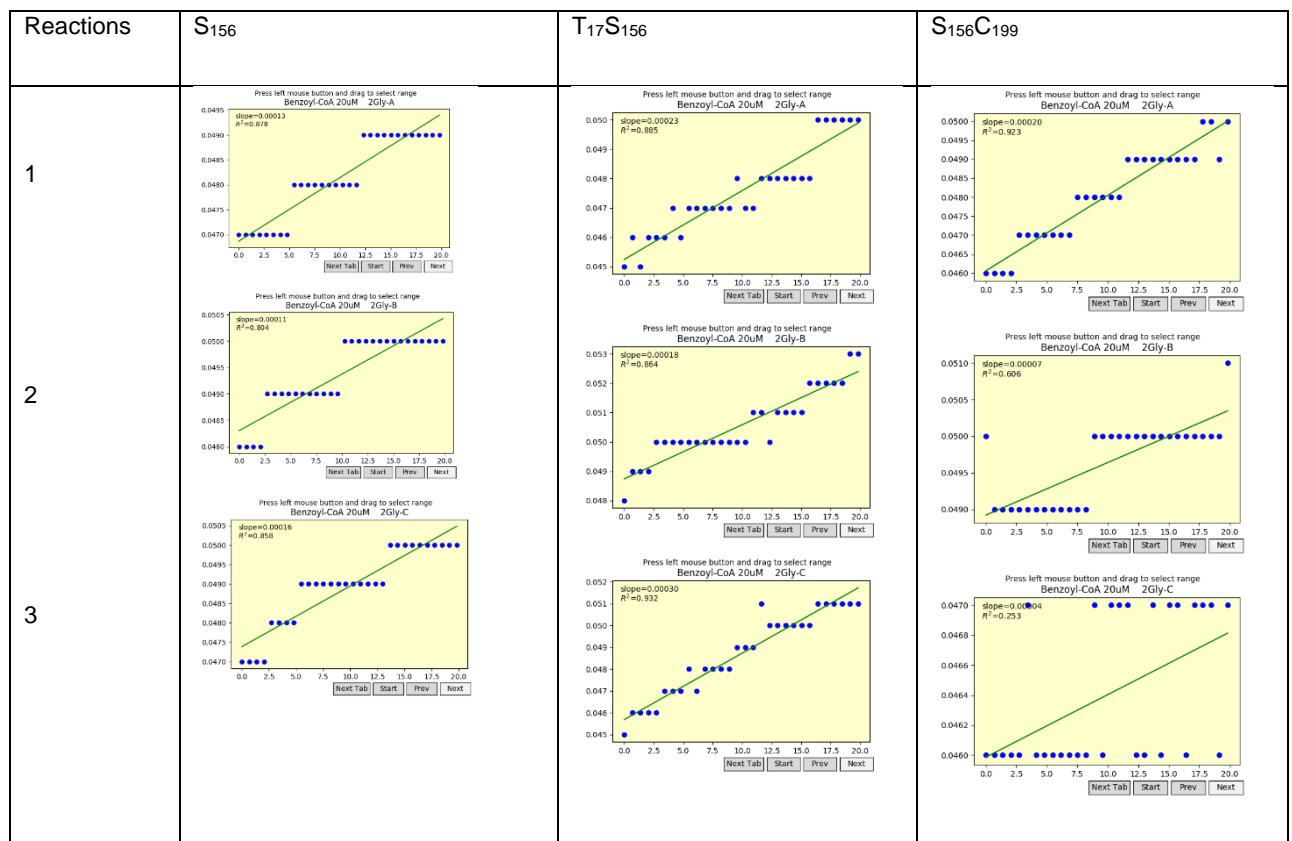
	11:37	0,053	0,054	0,053	0,000577	1,08
	12:18	0,054	0,054	0,054	0	0
	12:59	0,054	0,054	0,054	0	0
	13:40	0,054	0,054	0,054	0	0
	14:21	0,054	0,054	0,054	0	0
	15:02	0,055	0,055	0,055	0	0
	15:43	0,055	0,055	0,055	0	0
	16:24	0,055	0,055	0,055	0	0
	17:05	0,055	0,055	0,055	0	0
	17:46	0,055	0,055	0,055	0	0
	18:27	0,055	0,055	0,055	0	0
	19:08	0,055	0,055	0,055	0	0
	19:49	0,055	0,055	0,055	0	0

The raw data of the bi-substrate kinetics for all three proteins  $S_{156}$ ,  $T_{17}S_{156}$  and  $S_{156}C_{199}$ . The graphs are absorbance (y-axis) versus time (x-axis). After the graphs the raw triplicate data for all three variants with the different substrate concentrations are available. The data point that were outliers were removed before any kinetic data was done.

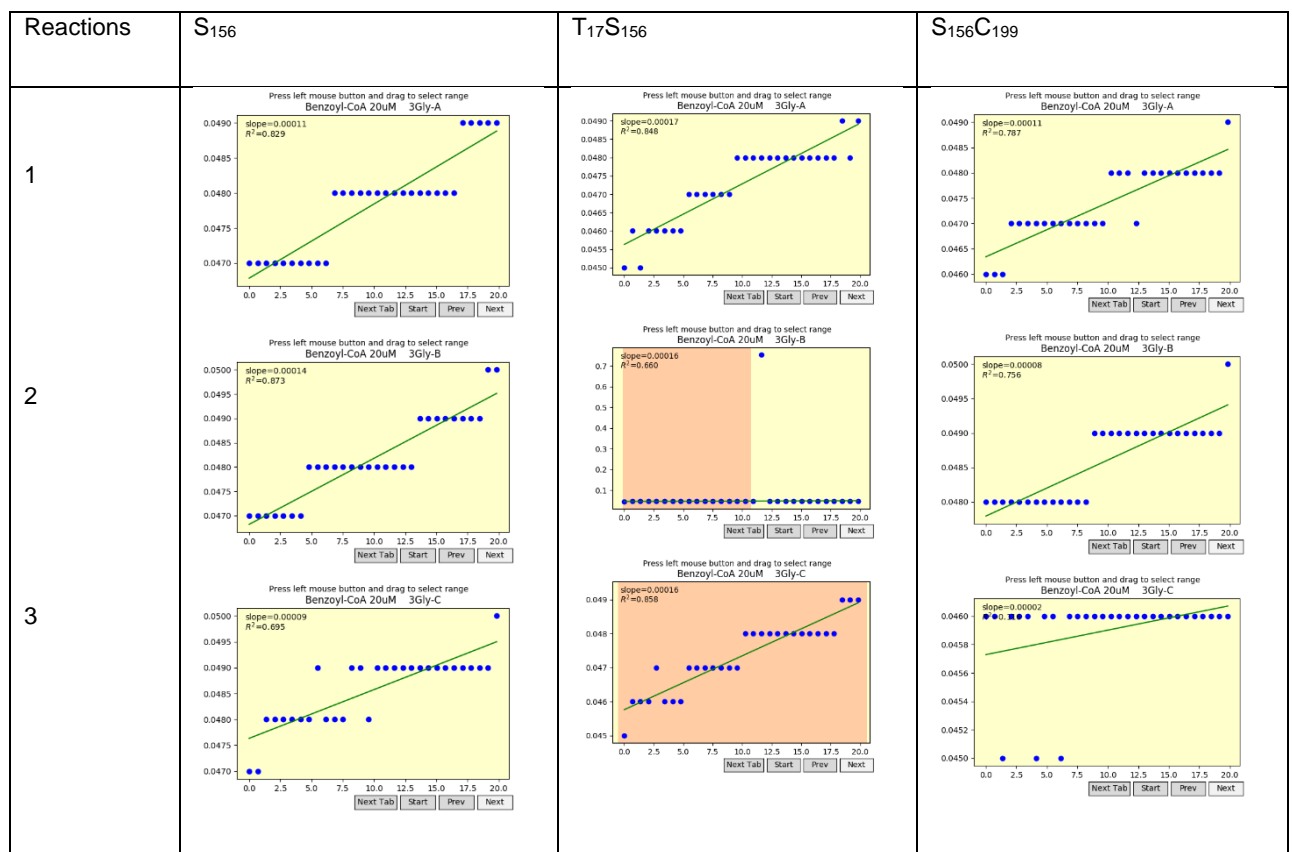
**Benzoyl-CoA : 20 $\mu$ M and Glycine 1mM**

Reactions	$S_{156}$	$T_{17}S_{156}$	$S_{156}C_{199}$
1			
2			
3			

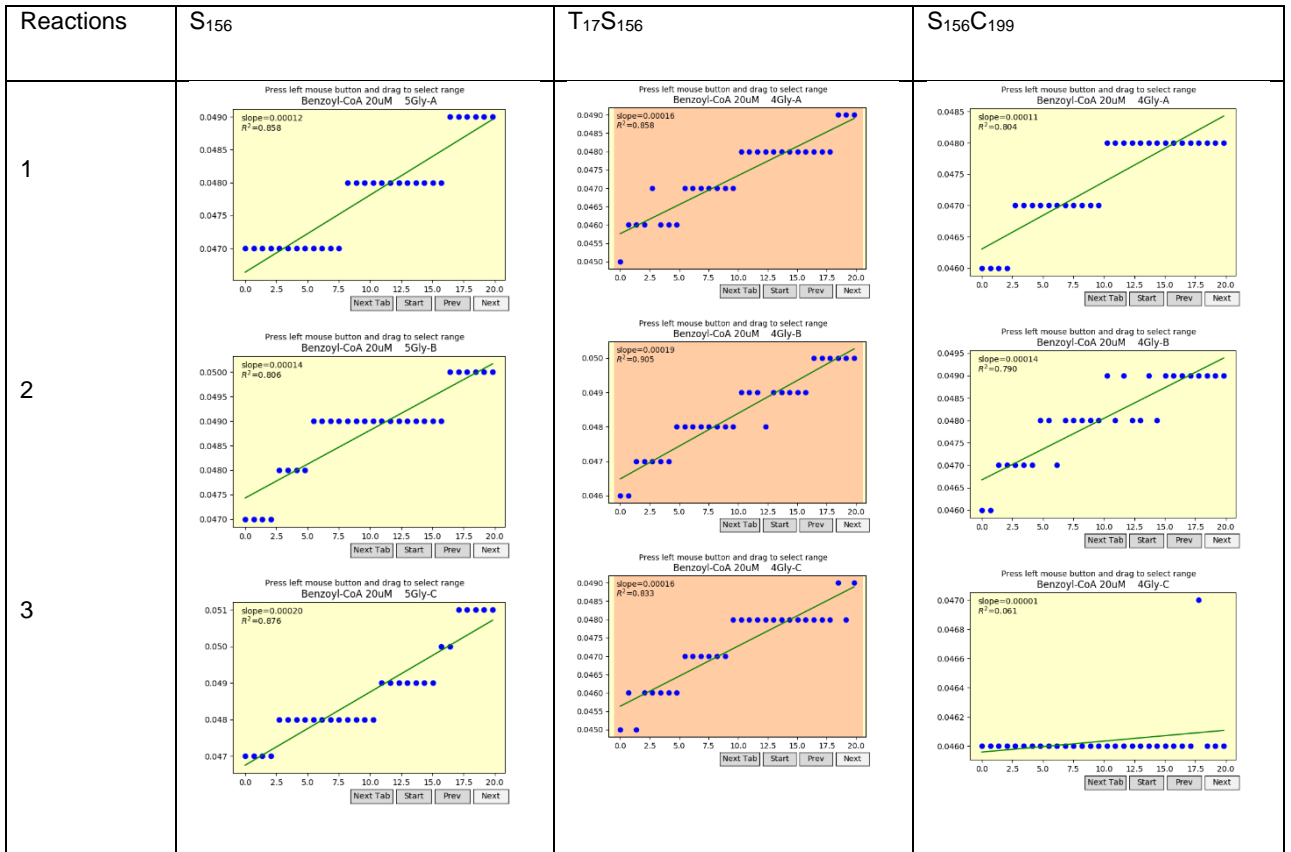
### Benzoyl-CoA : 20μM and Glycine 2mM



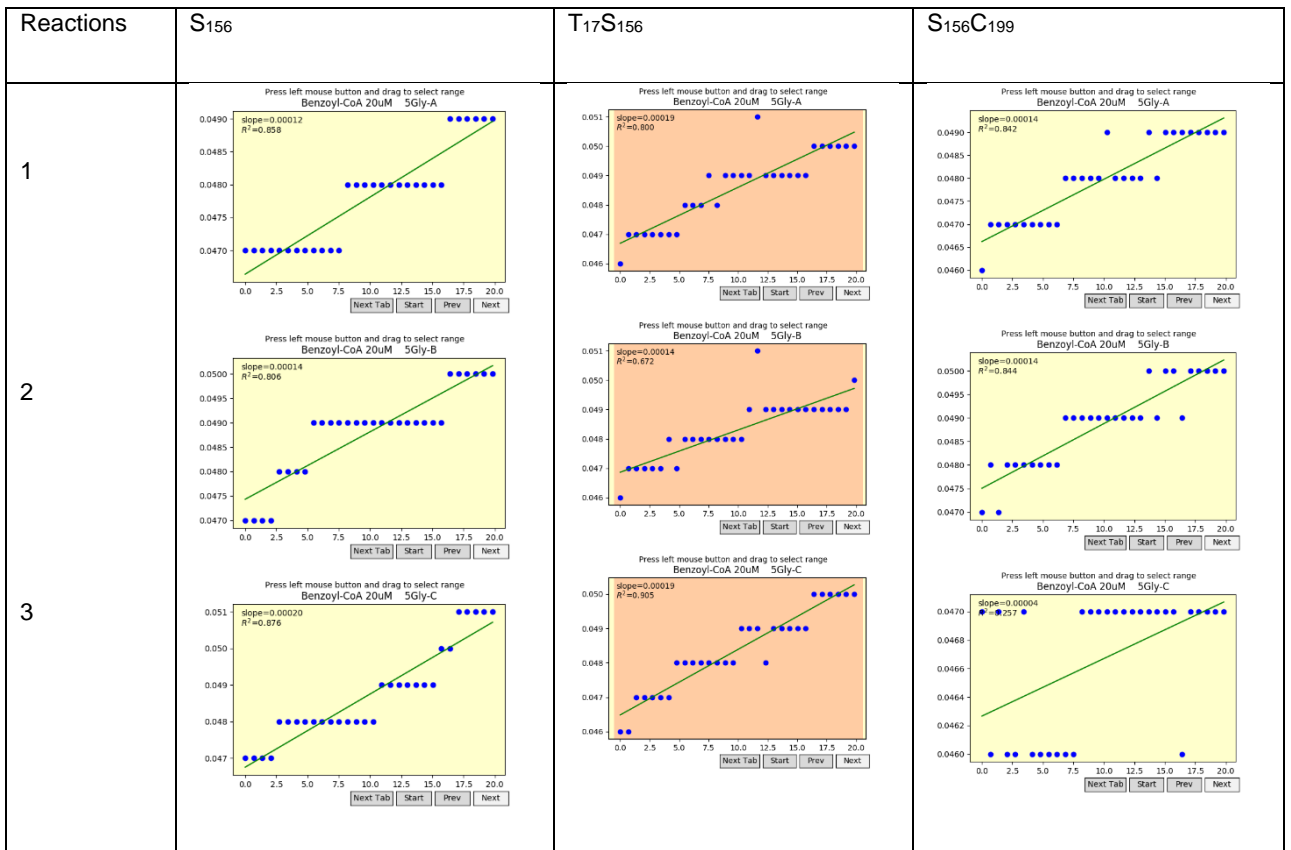
### Benzoyl-CoA : 20μM and Glycine 3mM



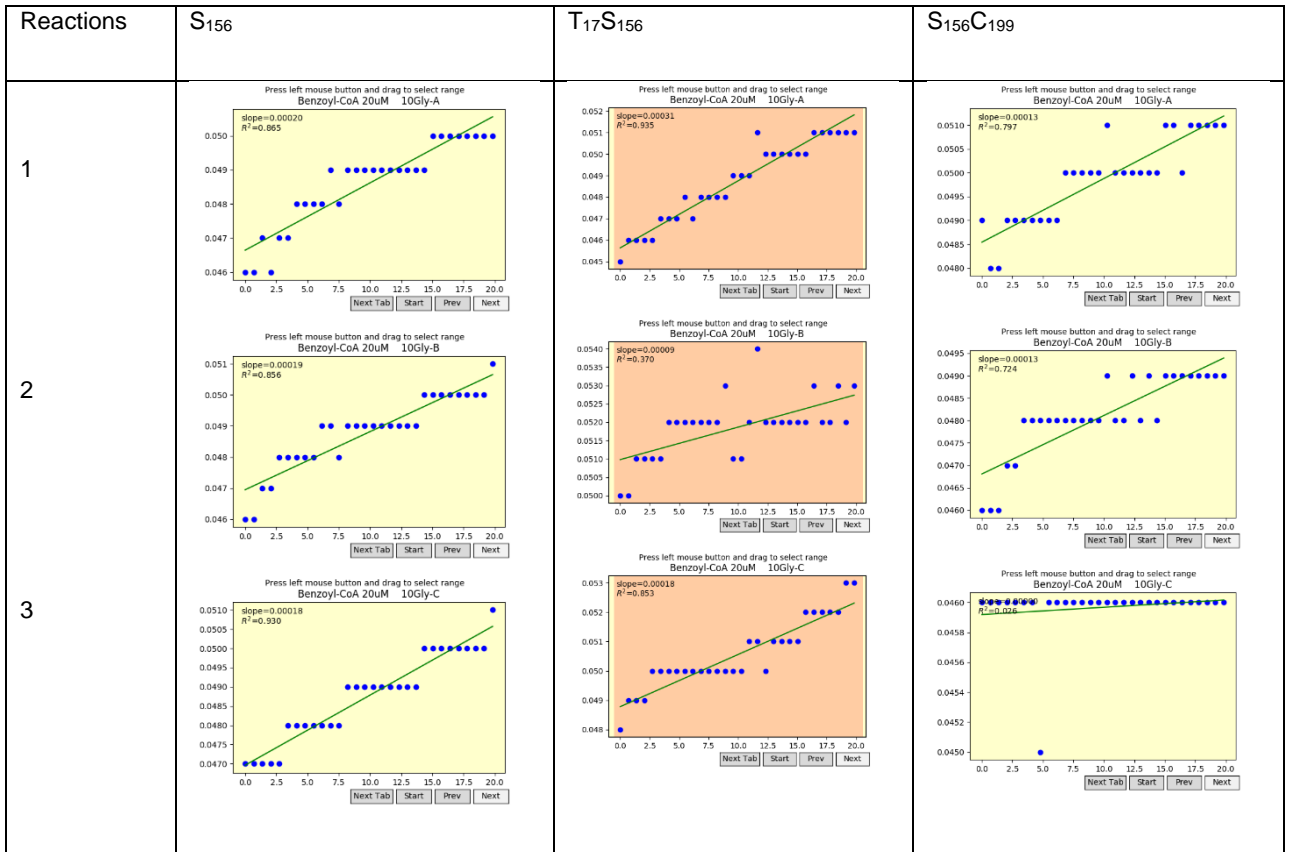
**Benzoyl-CoA : 20μM and Glycine 4mM**



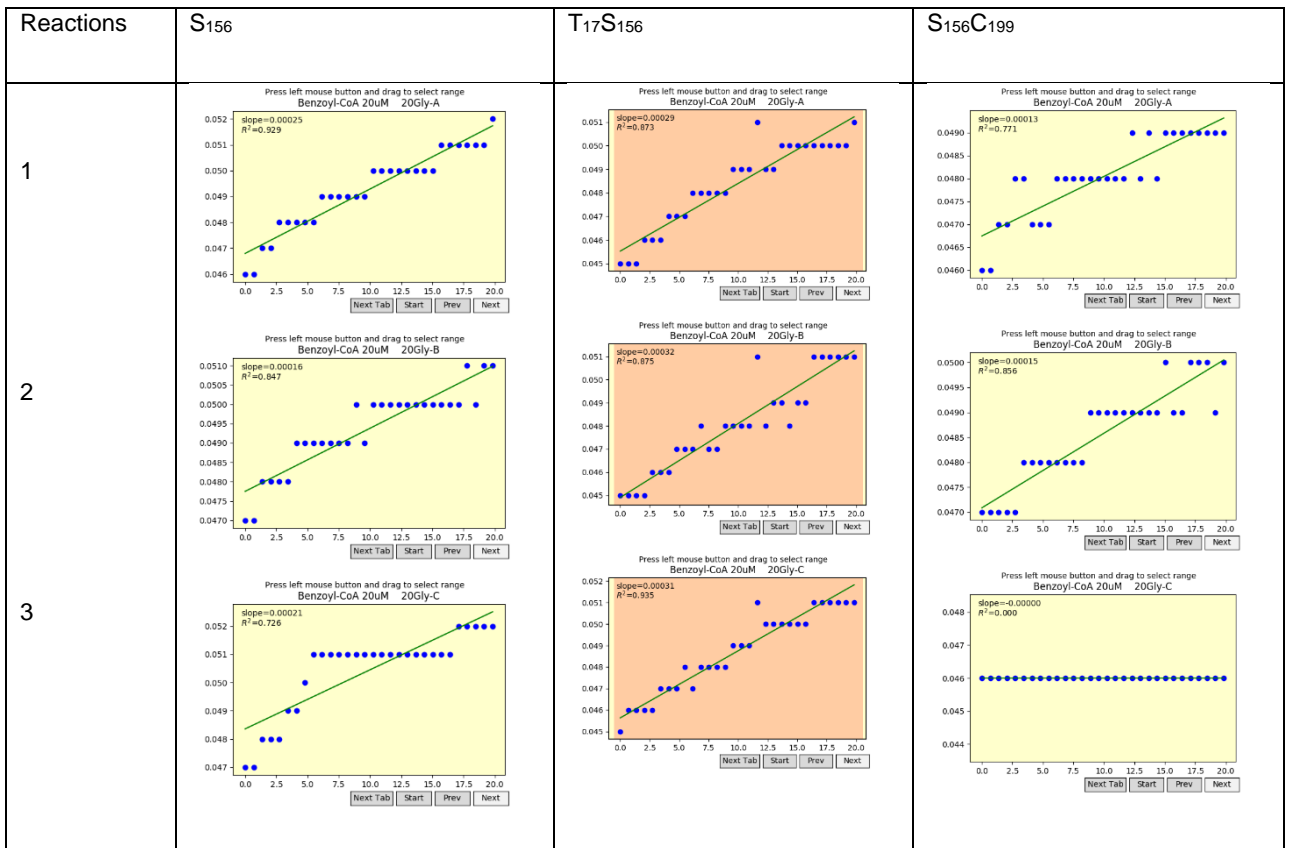
**Benzoyl-CoA : 20μM and Glycine 5mM**



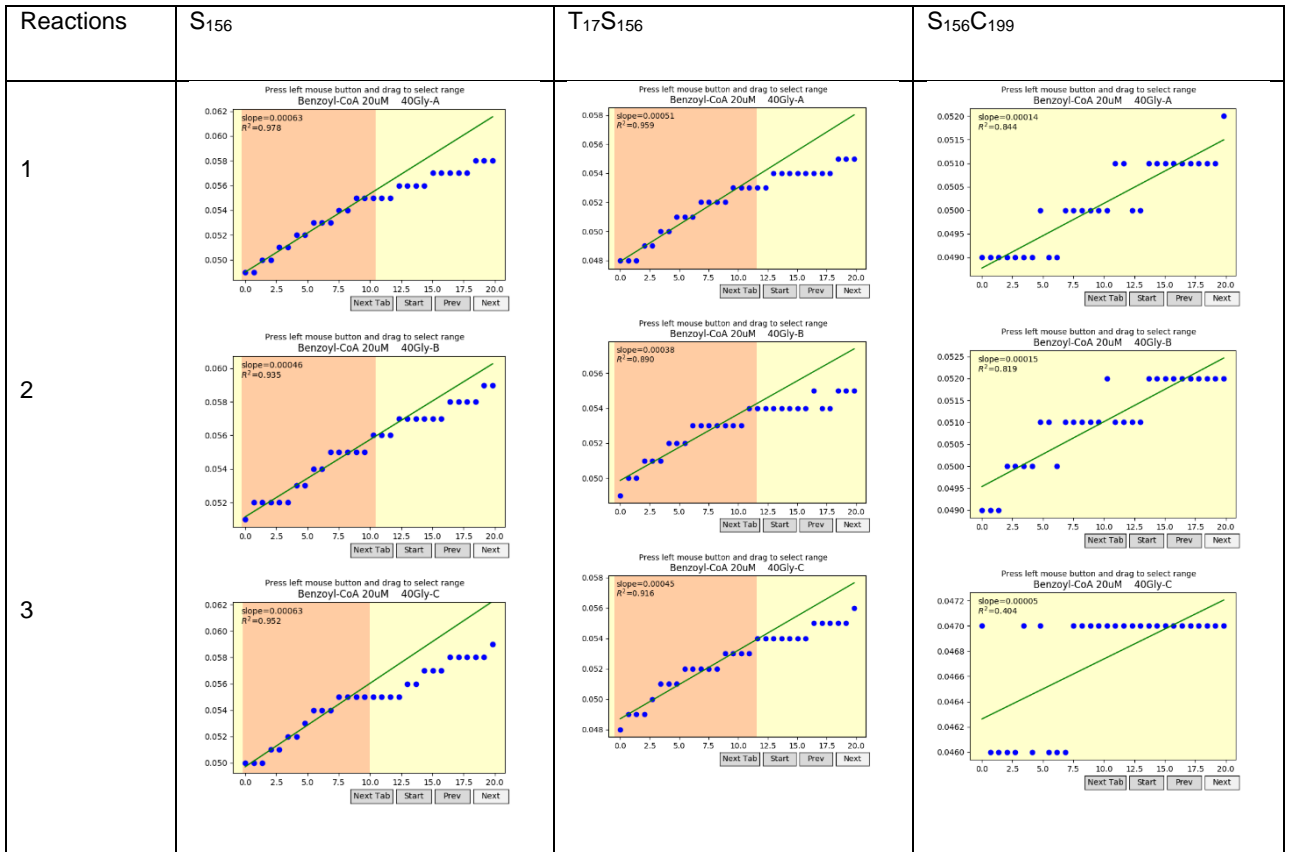
## Benzoyl-CoA : 20μM and Glycine 10mM



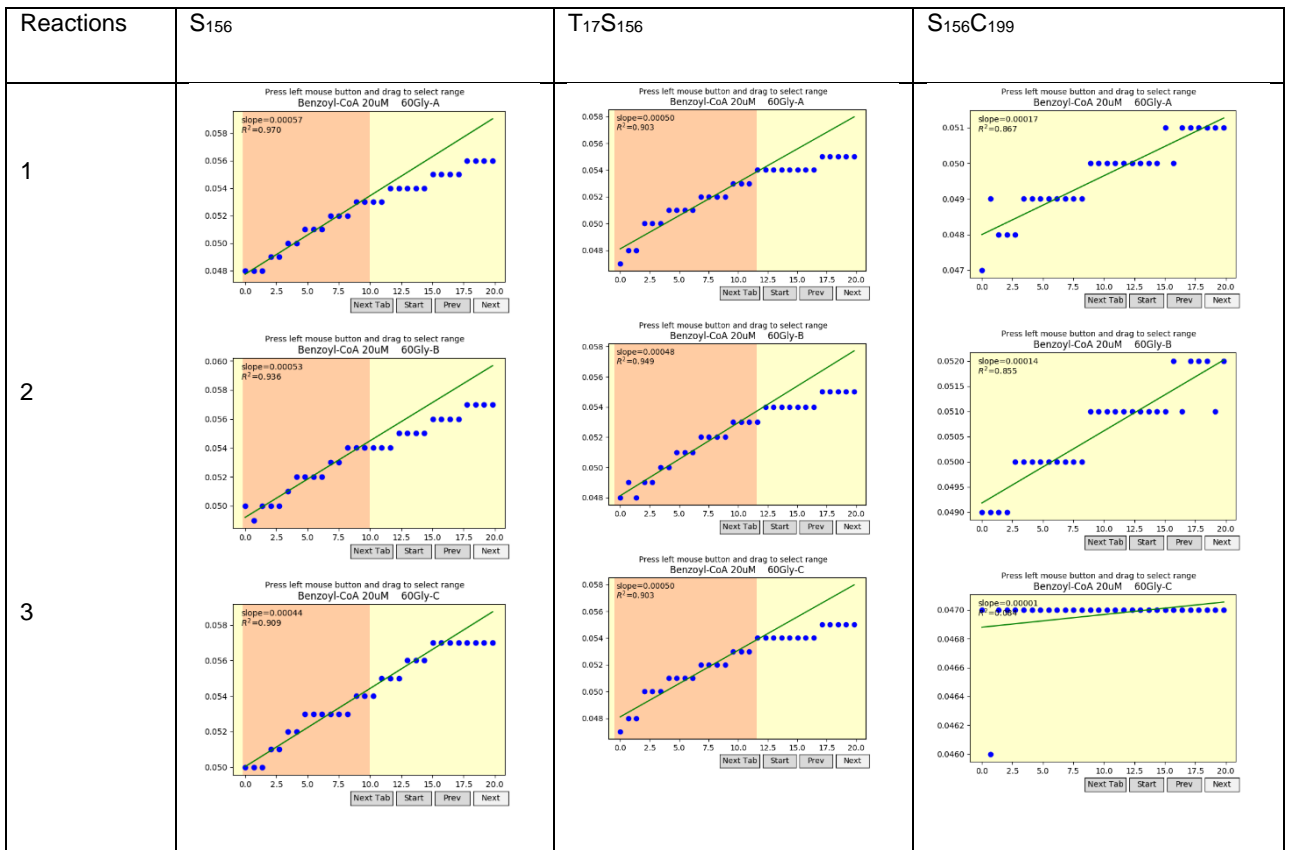
## Benzoyl-CoA : 20μM and Glycine 20mM



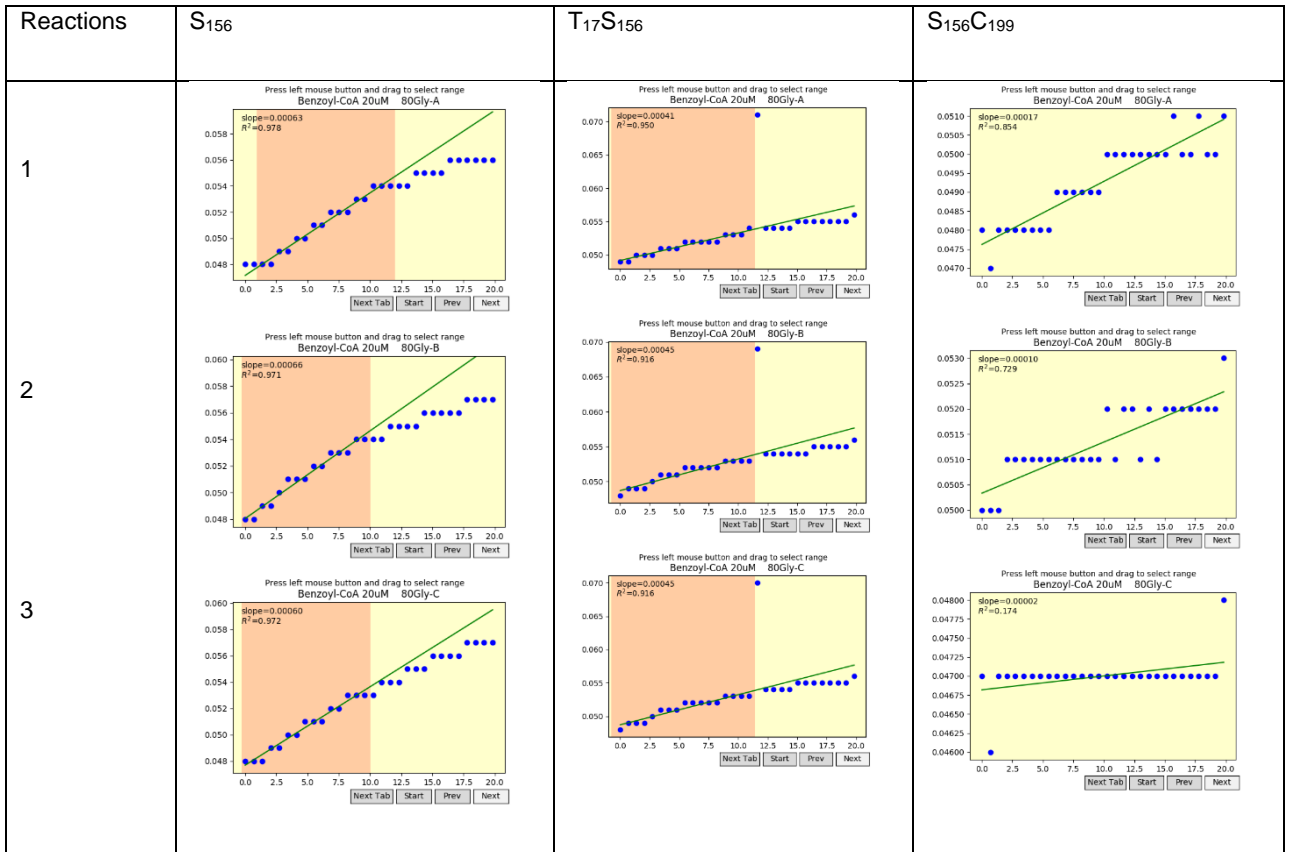
**Benzoyl-CoA : 20μM and Glycine 40mM**



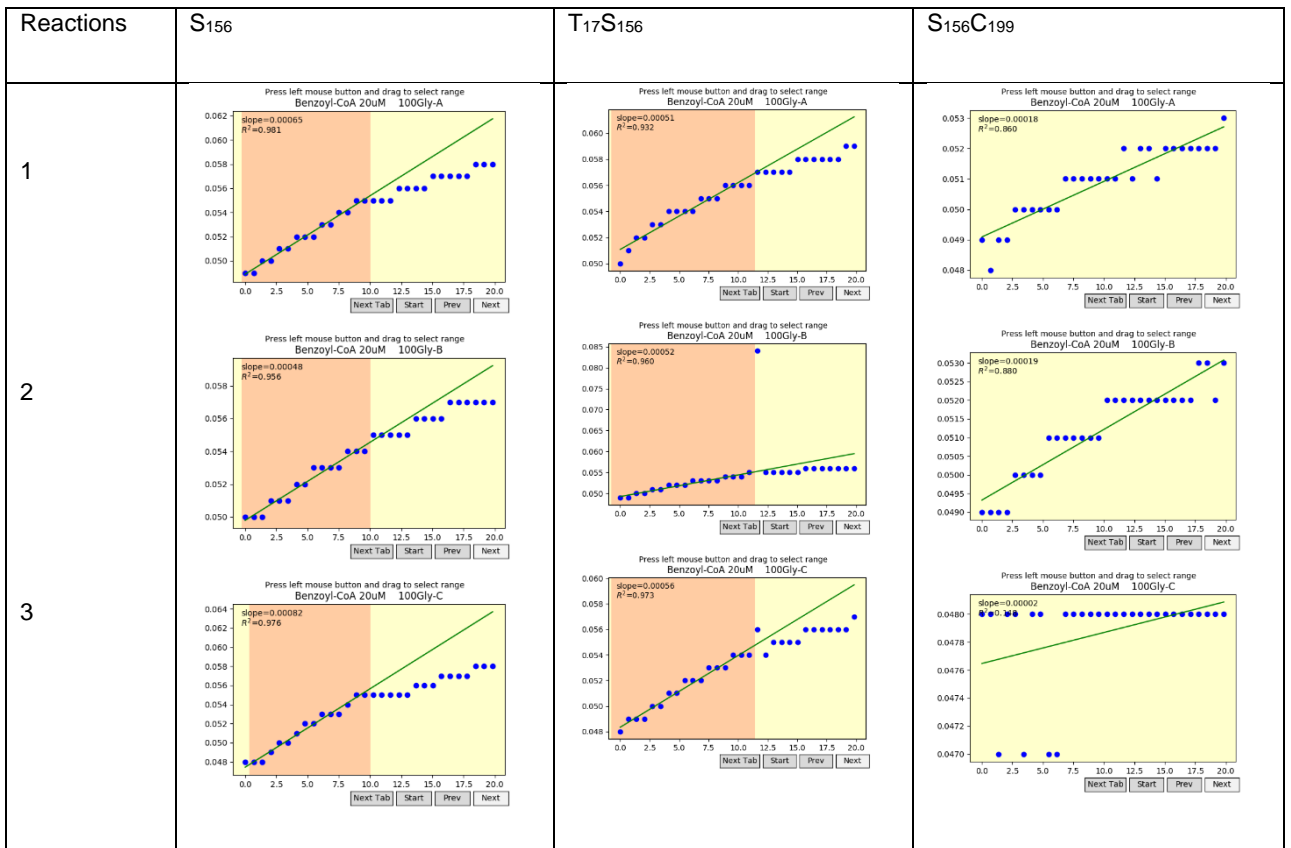
**Benzoyl-CoA : 20μM and Glycine 60mM**



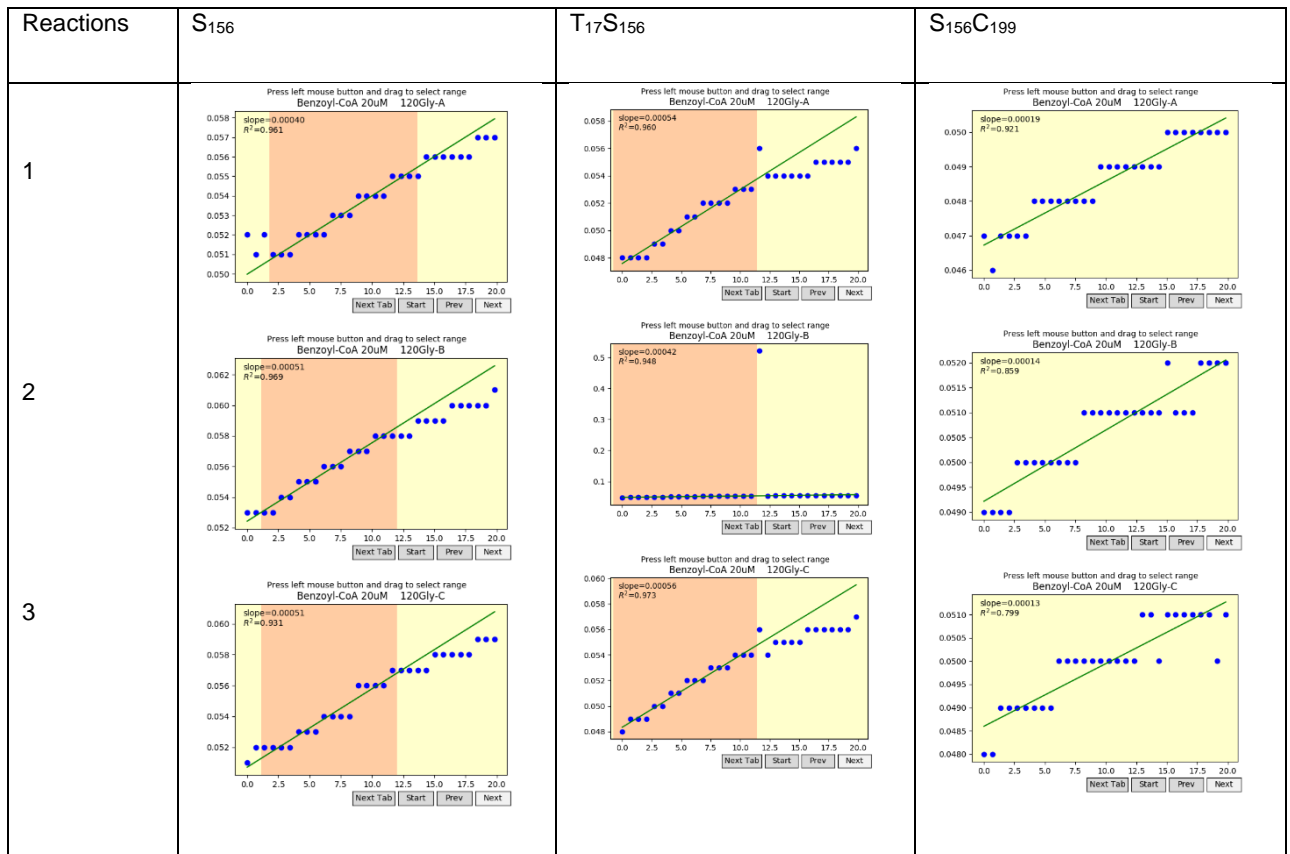
## Benzoyl-CoA : 20μM and Glycine 80mM



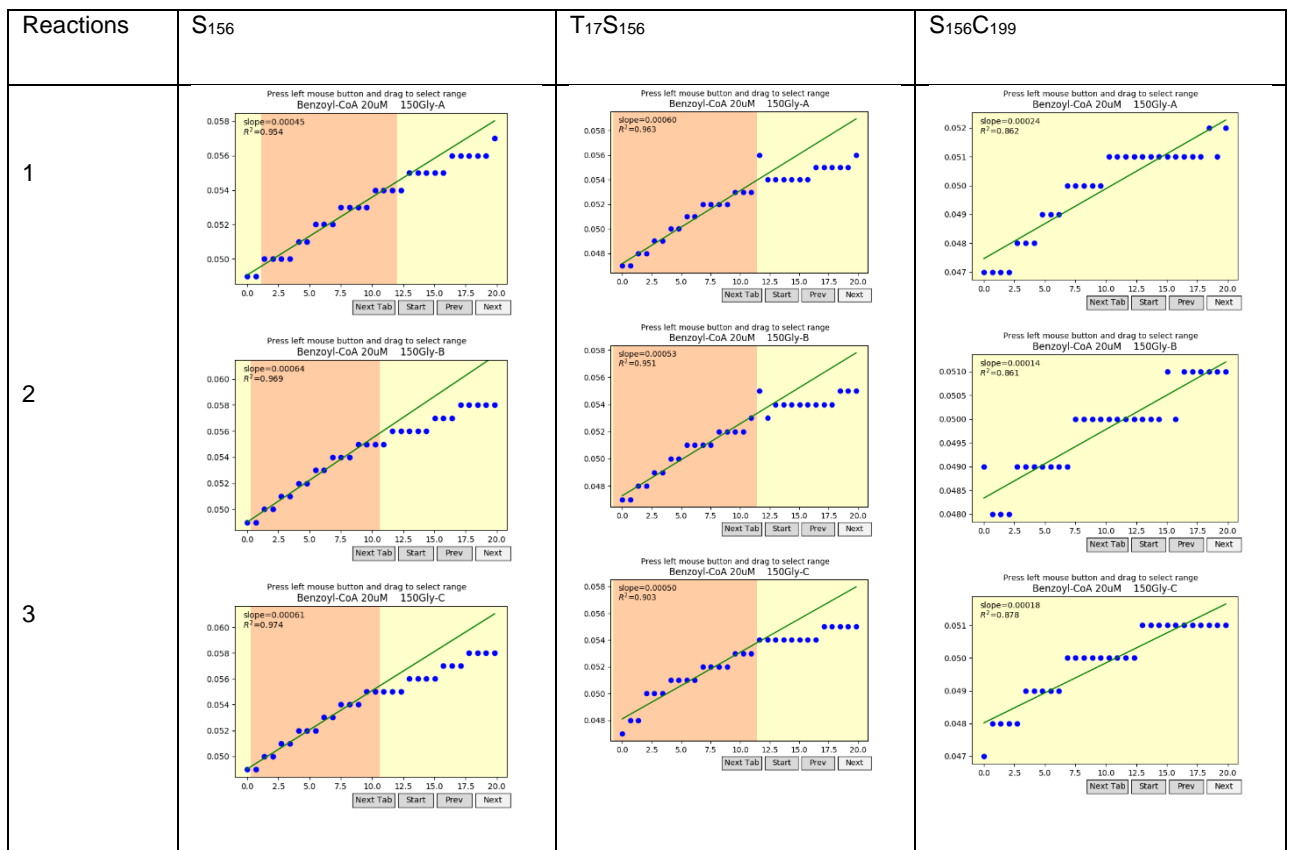
## Benzoyl-CoA : 20μM and Glycine 100mM



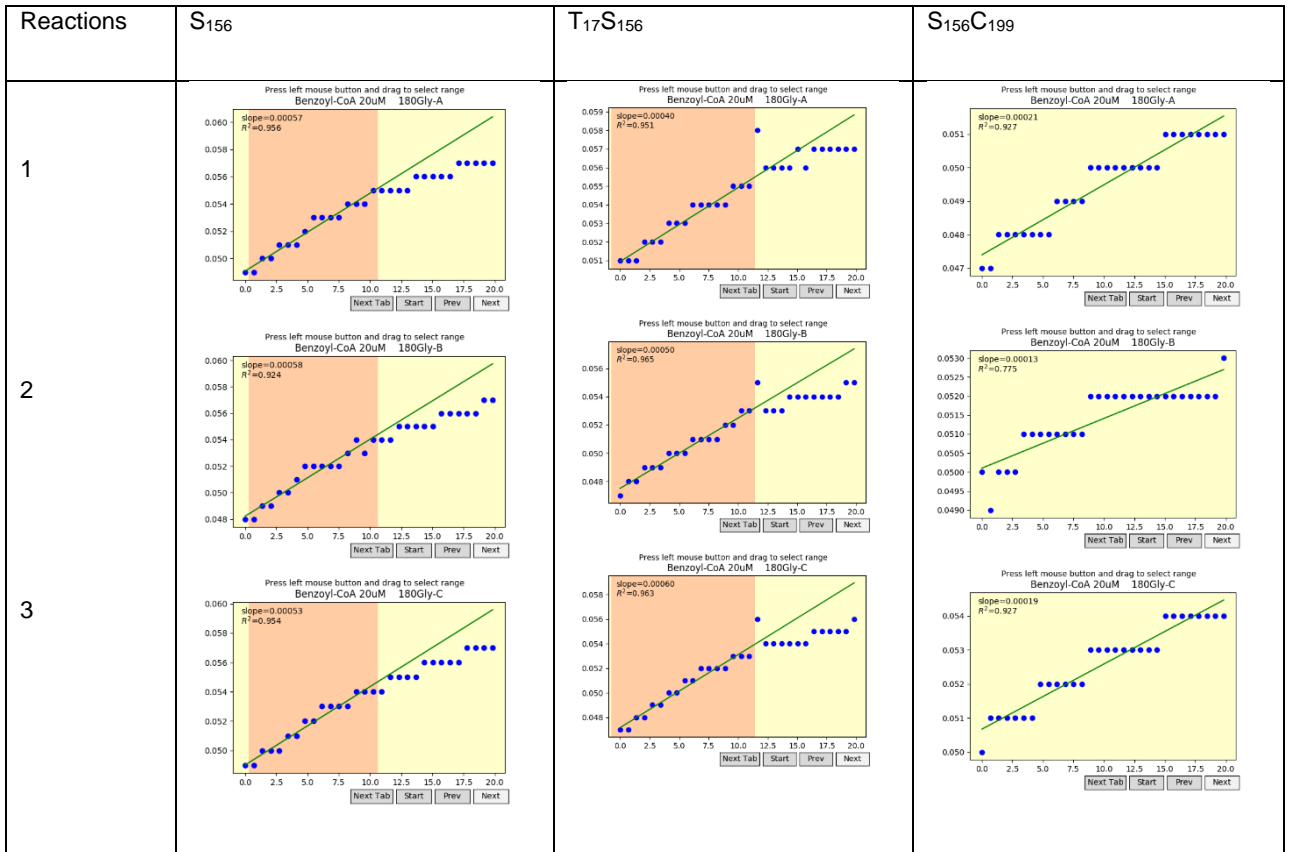
## Benzoyl-CoA : 20μM and Glycine 120mM



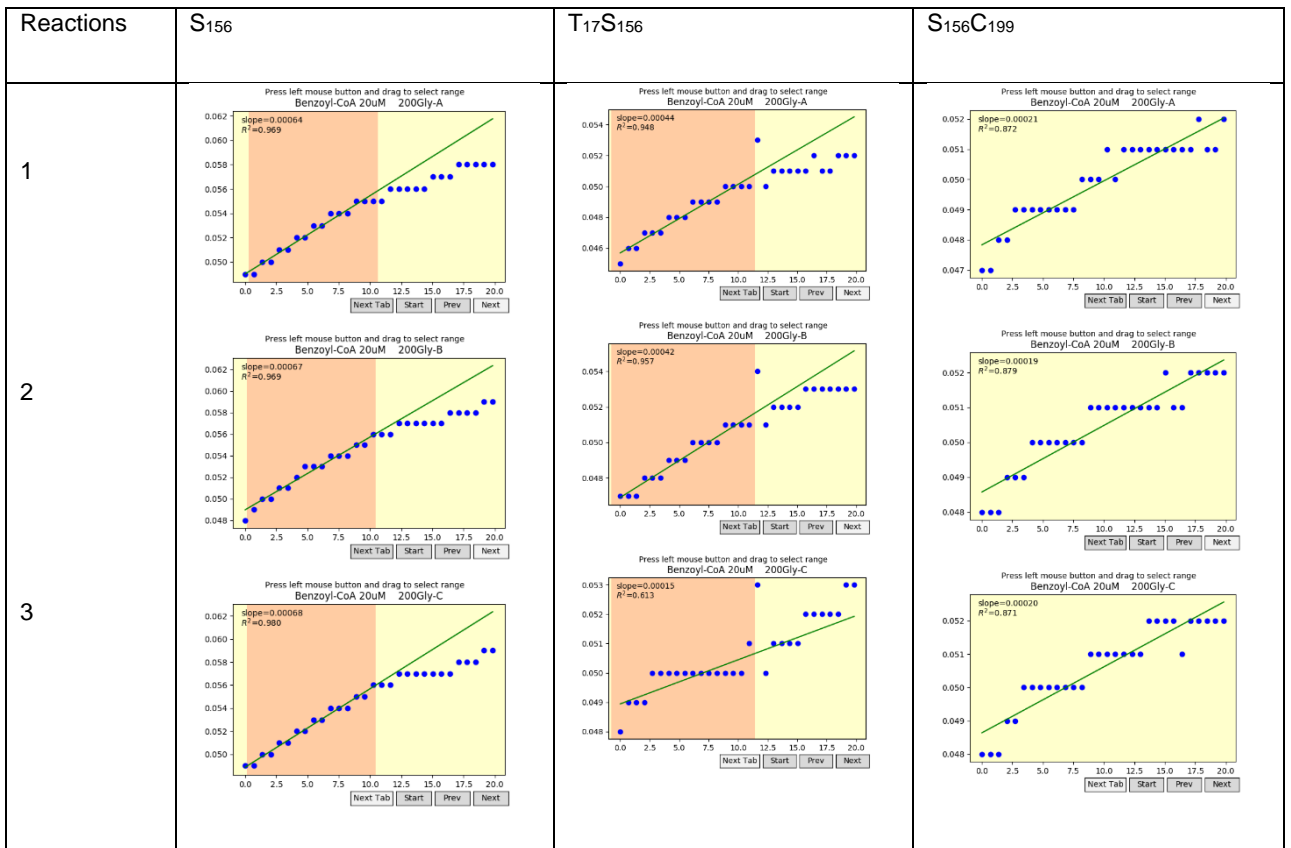
## Benzoyl-CoA : 20μM and Glycine 150mM



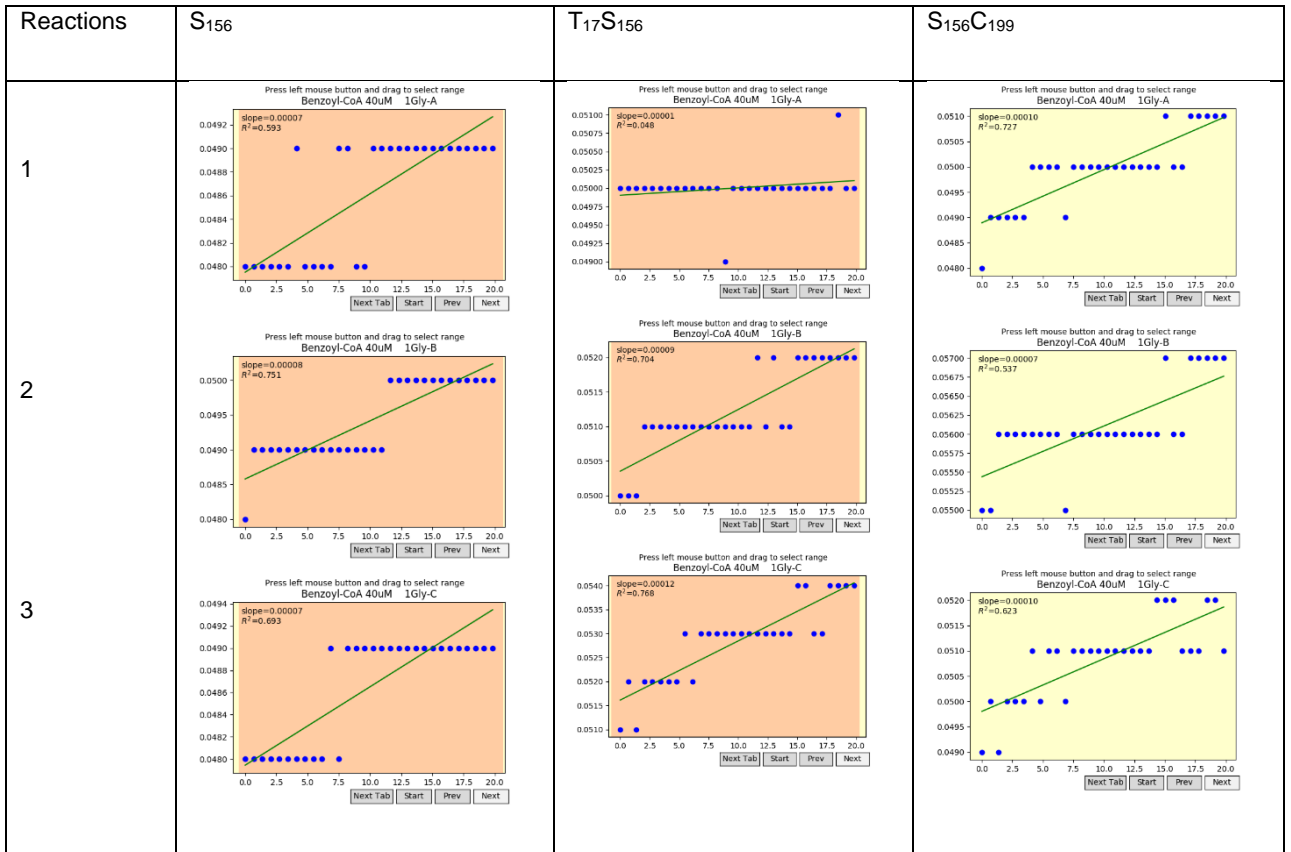
## Benzoyl-CoA : 20μM and Glycine 180mM



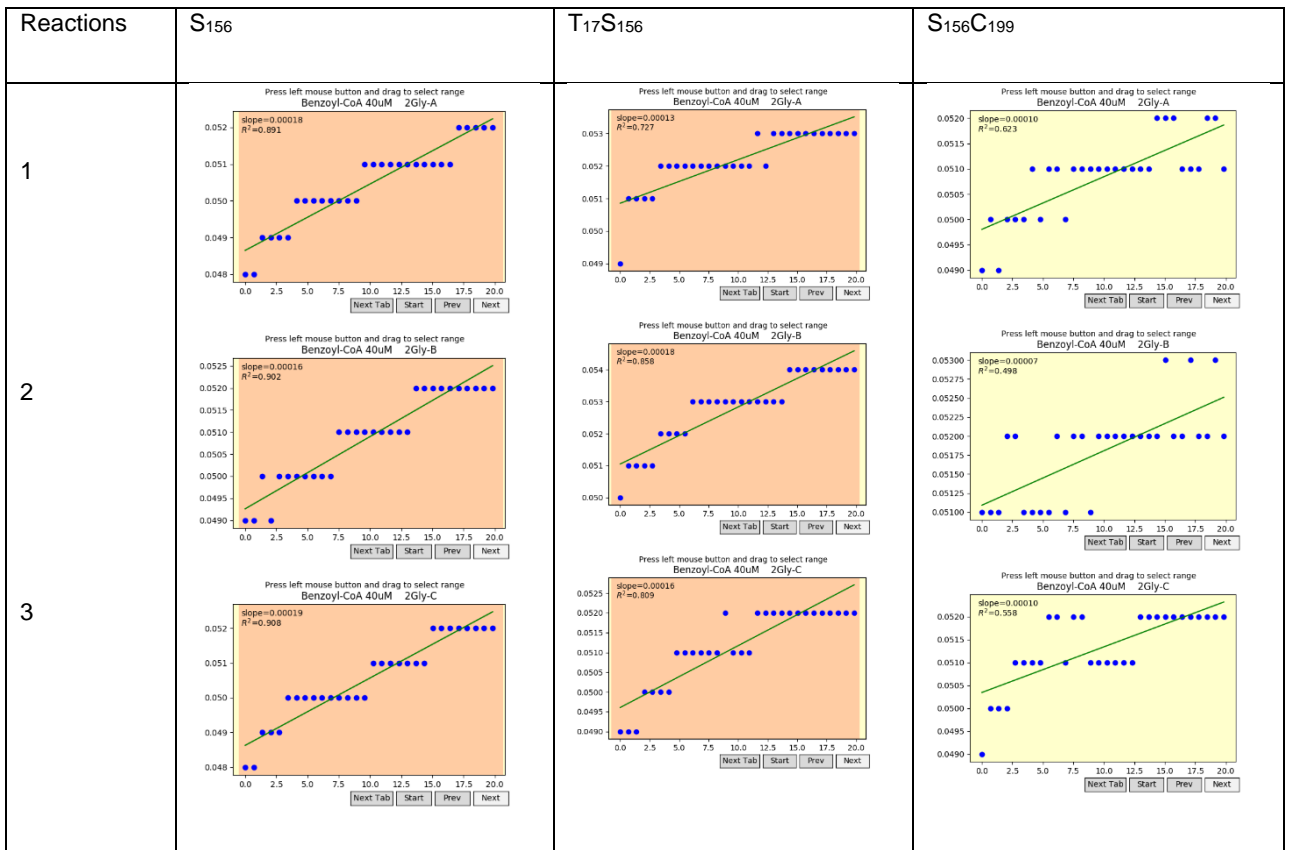
## Benzoyl-CoA : 20μM and Glycine 200mM



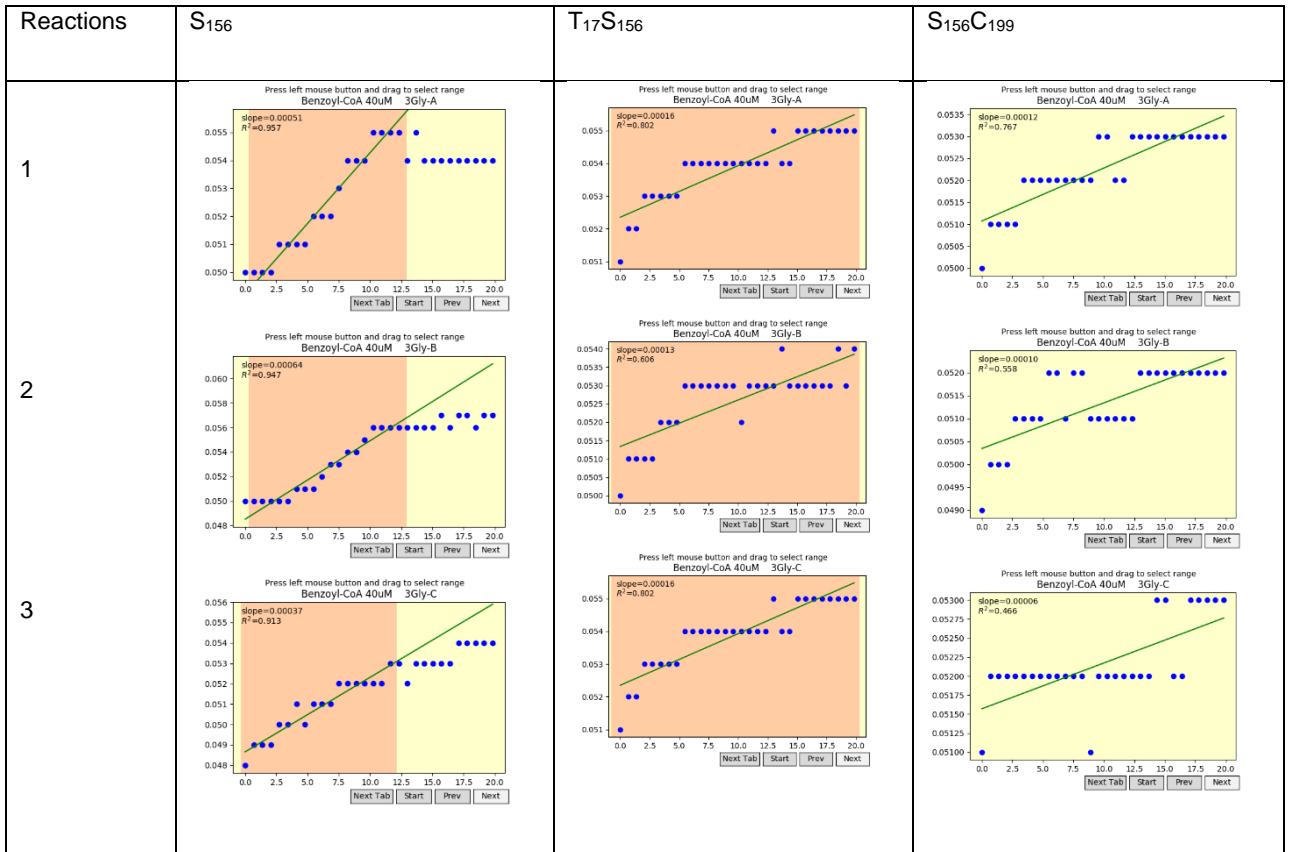
### Benzoyl-CoA : 40μM and Glycine 1mM



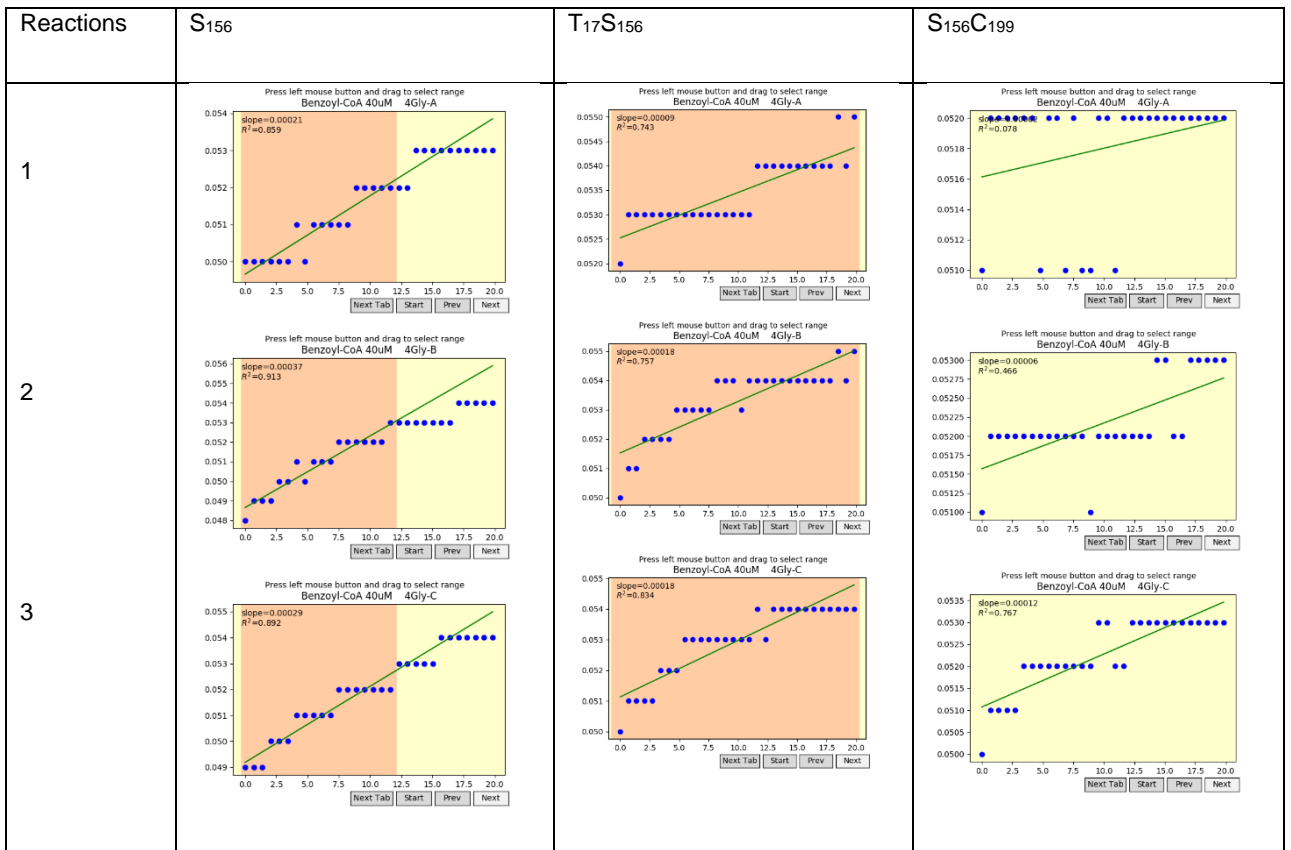
### Benzoyl-CoA : 40μM and Glycine 2mM



**Benzoyl-CoA : 20µM and Glycine 3mM**



**Benzoyl-CoA : 40µM and Glycine 4mM**



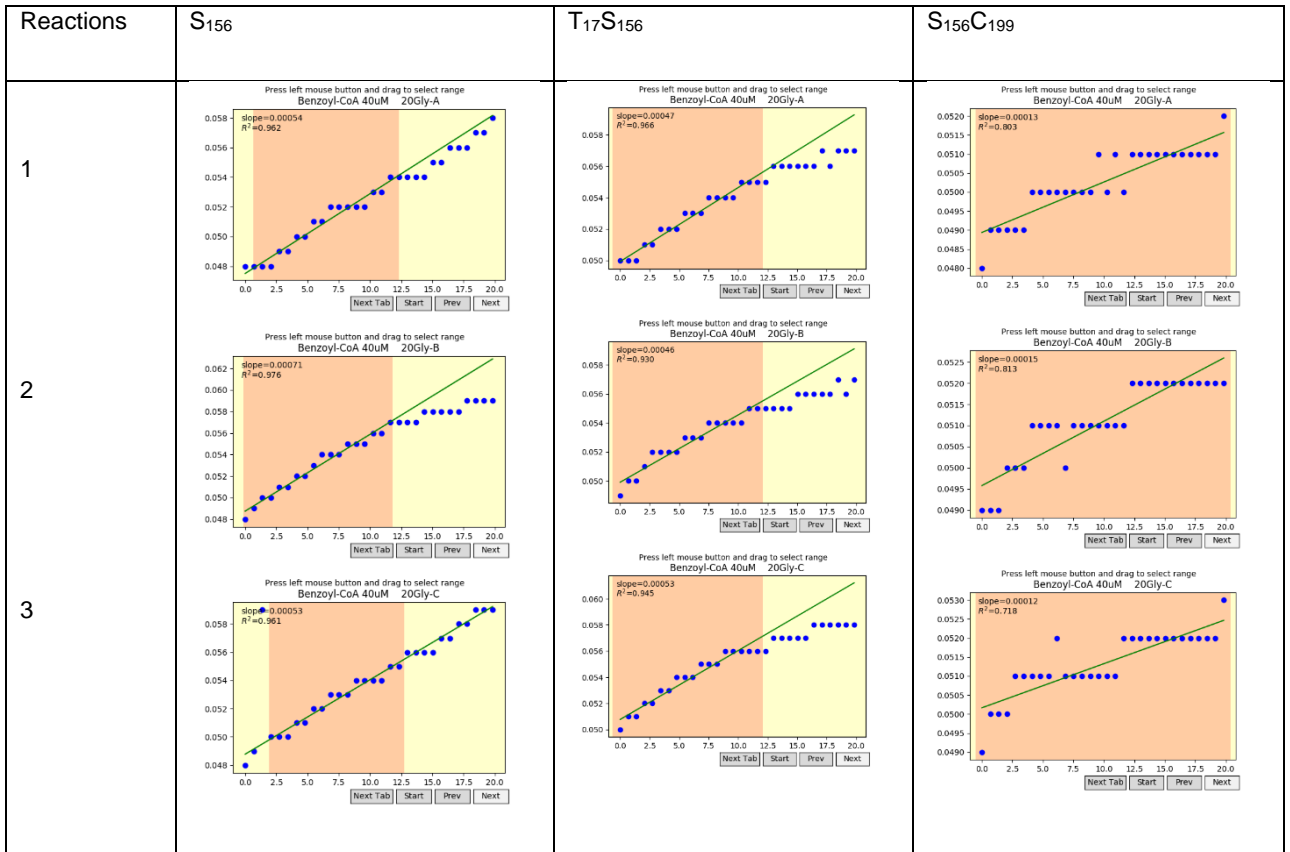
# Benzoyl-CoA : 40μM and Glycine 5mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-A slope=0.00044 R<sup>2</sup>=0.893</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-A slope=0.00022 R<sup>2</sup>=0.802</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-A slope=0.00006 R<sup>2</sup>=0.574</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-B slope=0.00032 R<sup>2</sup>=0.939</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-B slope=0.00019 R<sup>2</sup>=0.852</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-B slope=0.00012 R<sup>2</sup>=0.825</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-C slope=0.00045 R<sup>2</sup>=0.951</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-C slope=0.00019 R<sup>2</sup>=0.893</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 5Gly-C slope=0.00000 R<sup>2</sup>=0.004</p>

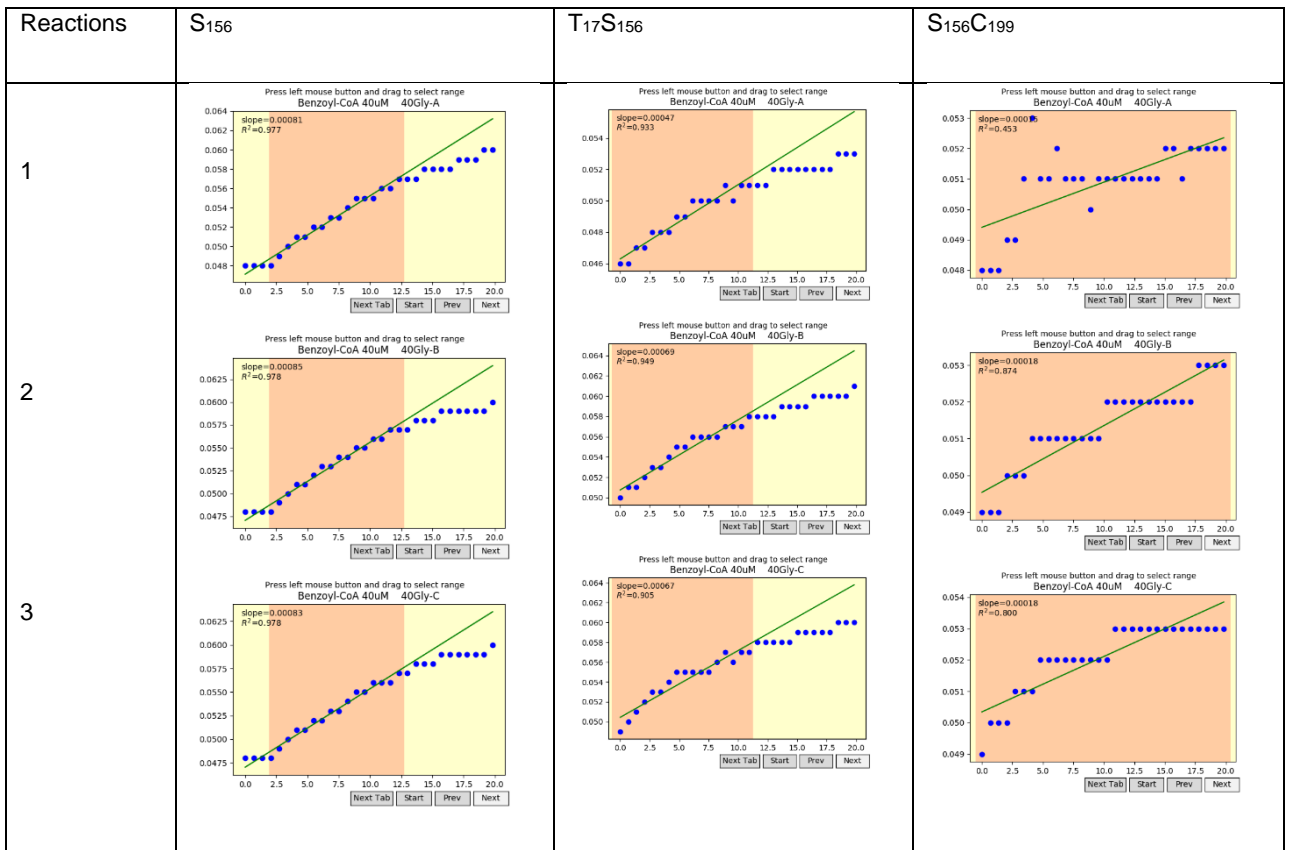
# Benzoyl-CoA : 40μM and Glycine 10mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 20Gly-A slope=0.00054 R<sup>2</sup>=0.962</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 10Gly-A slope=0.00034 R<sup>2</sup>=0.921</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 10Gly-A slope=0.00014 R<sup>2</sup>=0.874</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 20Gly-B slope=0.00071 R<sup>2</sup>=0.976</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 10Gly-B slope=0.00042 R<sup>2</sup>=0.926</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 10Gly-B slope=0.00016 R<sup>2</sup>=0.899</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 20Gly-C slope=0.00053 R<sup>2</sup>=0.961</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 10Gly-C slope=0.00037 R<sup>2</sup>=0.918</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40uM 10Gly-C slope=0.00012 R<sup>2</sup>=0.759</p>

**Benzoyl-CoA : 40μM and Glycine 20mM**



**Benzoyl-CoA : 40μM and Glycine 40mM**



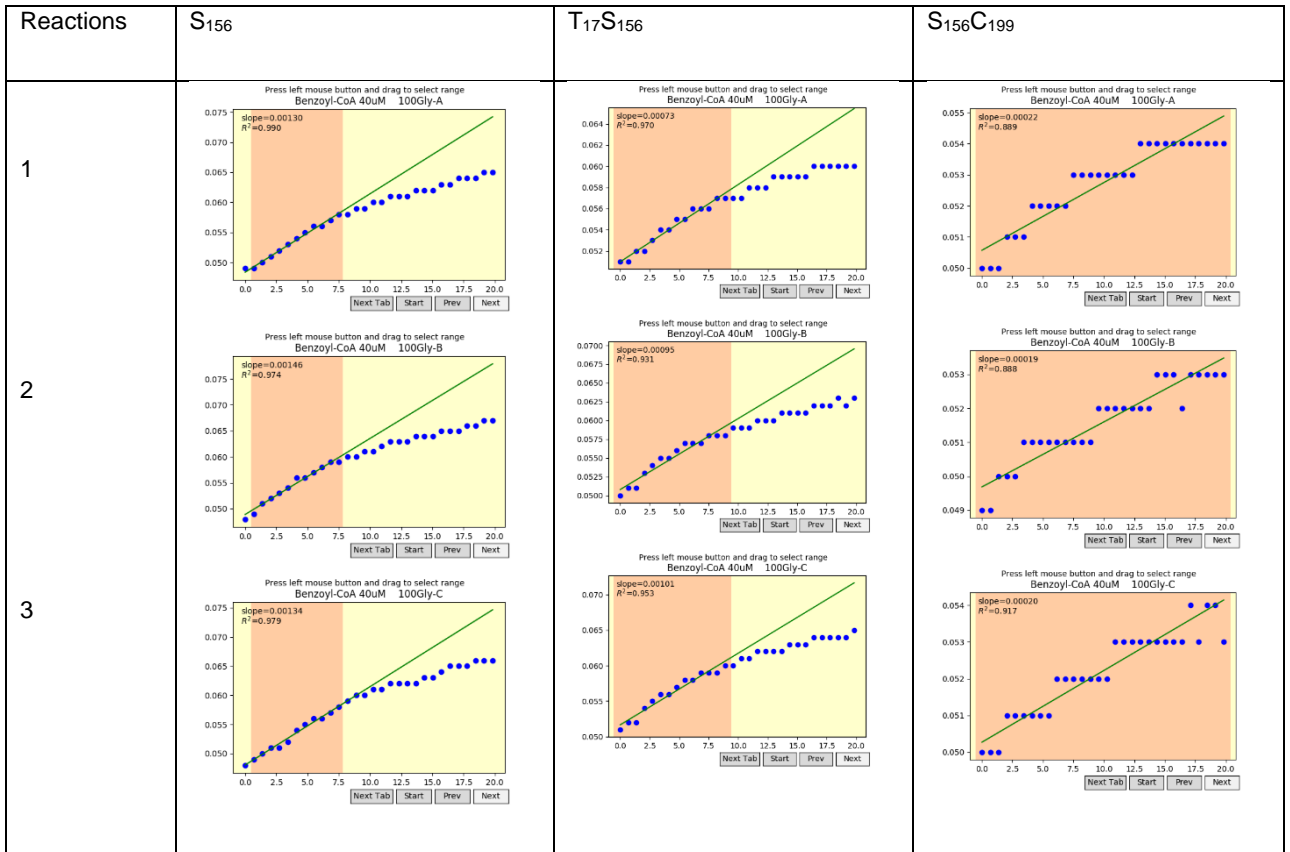
### Benzoyl-CoA : 40μM and Glycine 60mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 60Gly-C</p>

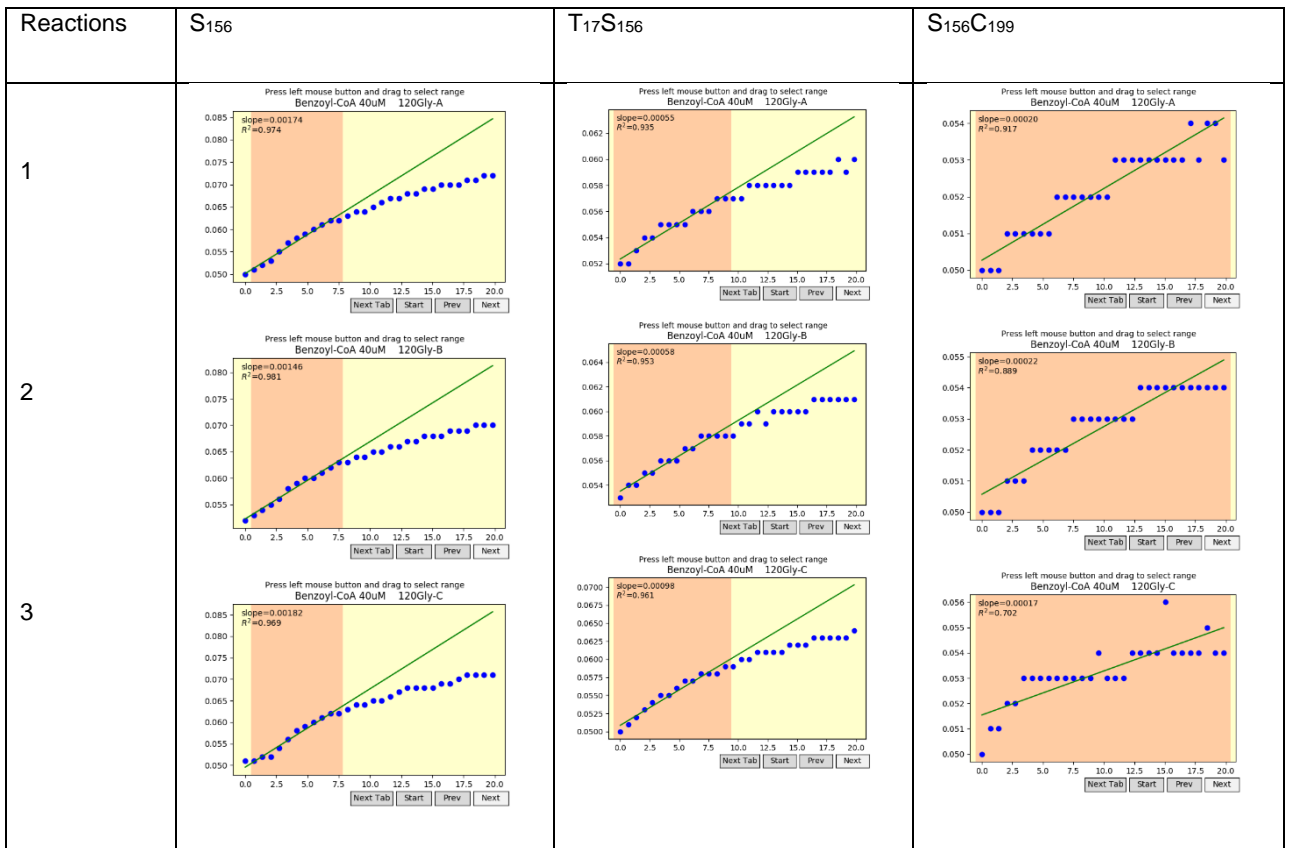
### Benzoyl-CoA : 40μM and Glycine 80mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 80Gly-C</p>

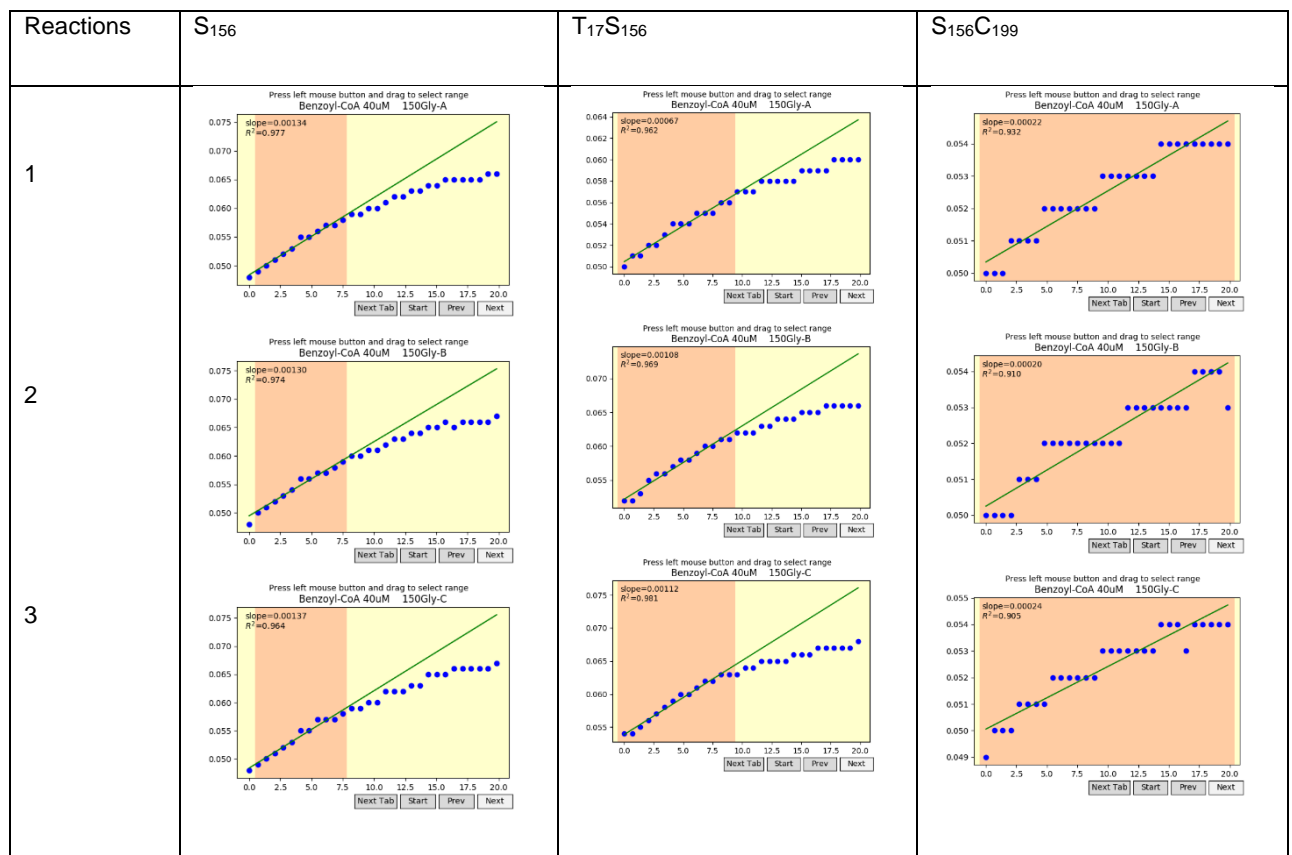
## Benzoyl-CoA : 40μM and Glycine 100mM



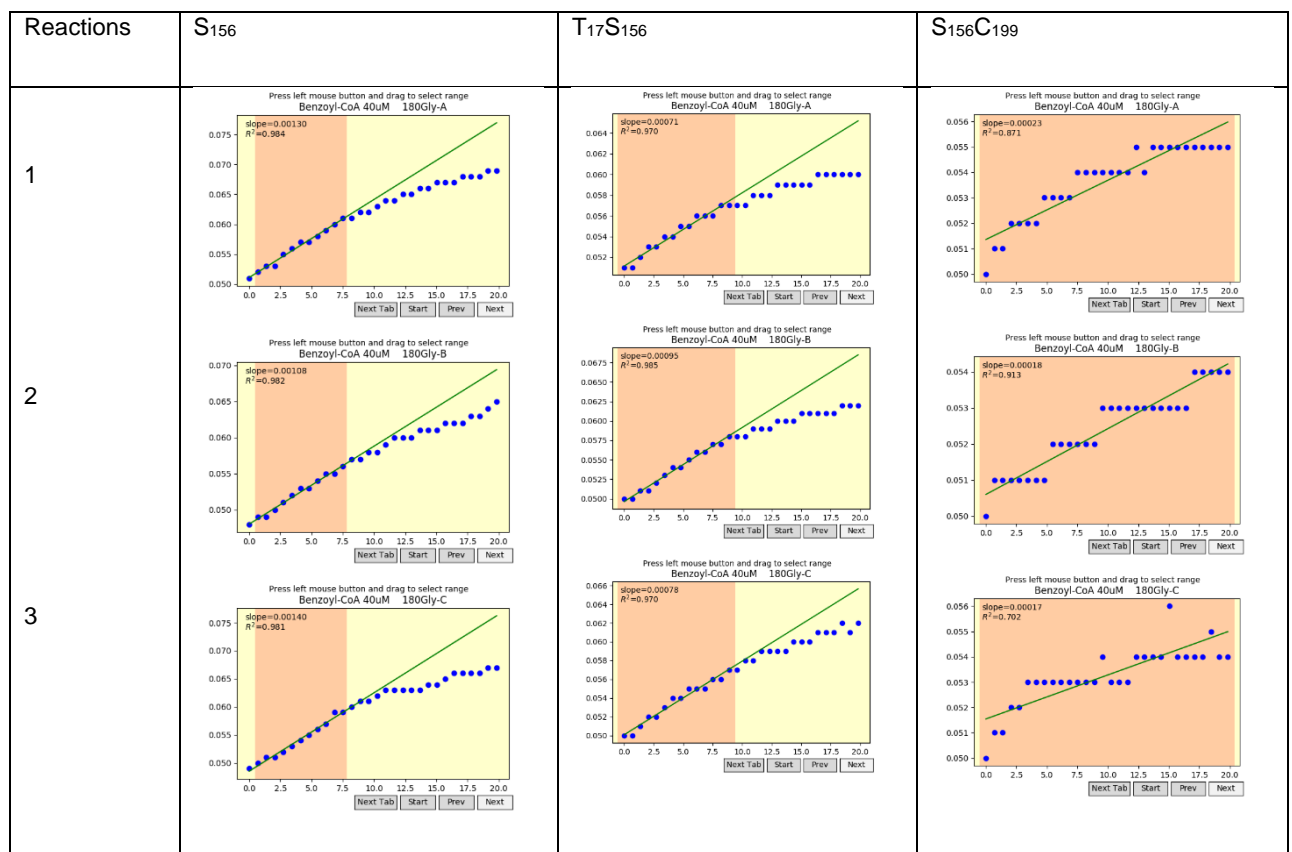
## Benzoyl-CoA : 40μM and Glycine 120mM



## Benzoyl-CoA : 40μM and Glycine 150mM



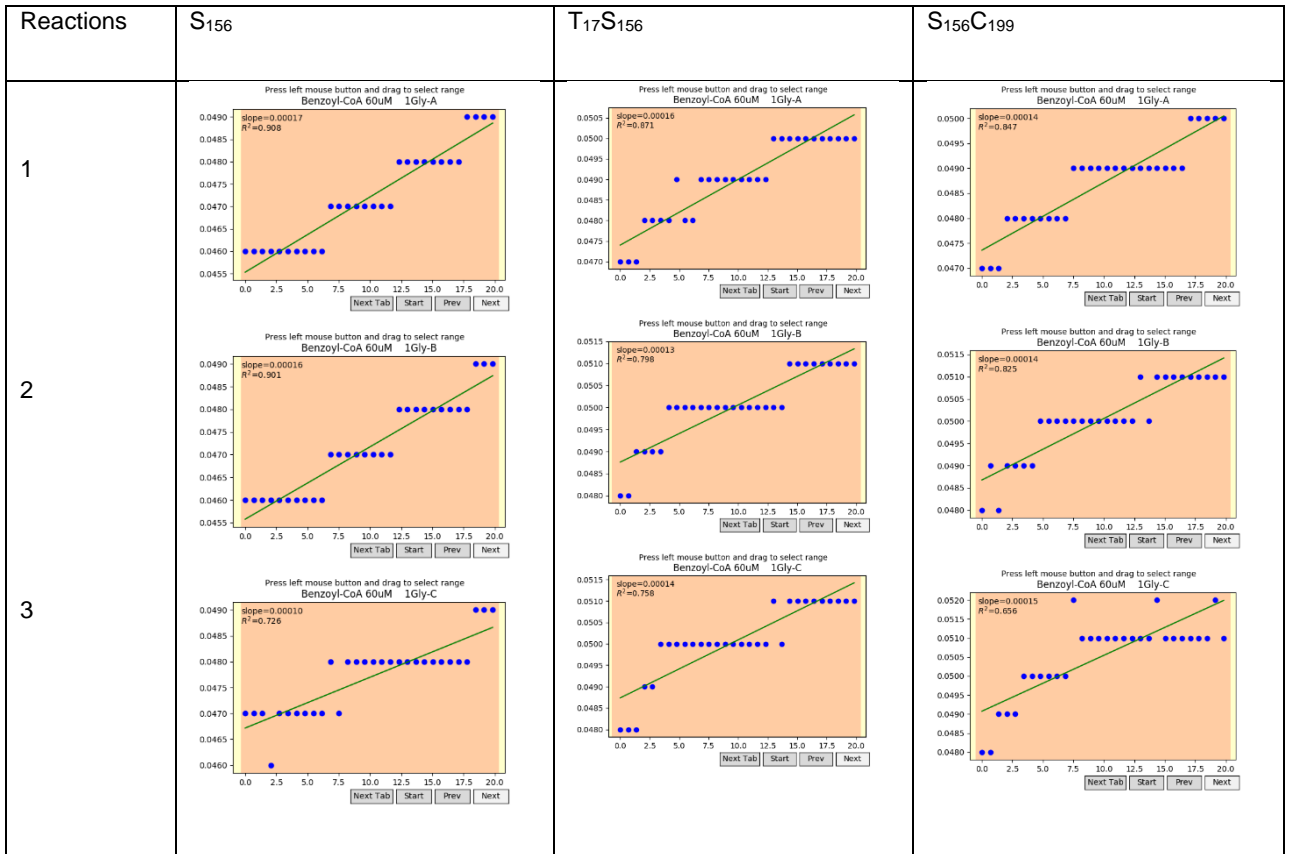
## Benzoyl-CoA : 40μM and Glycine 180mM



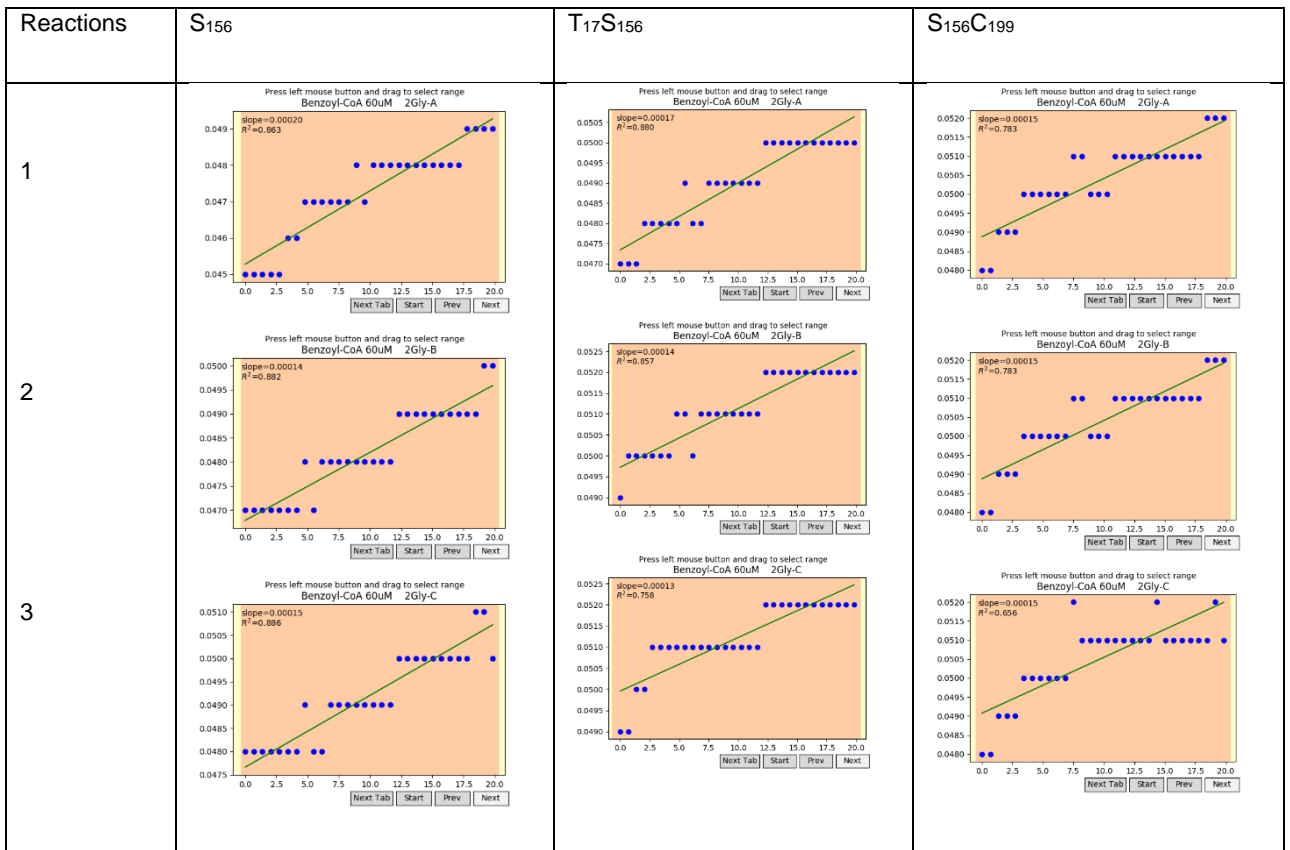
# Benzoyl-CoA : 40μM and Glycine 200mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-A</p> <p>slope=0.00162 R<sup>2</sup>=0.983</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-A</p> <p>slope=0.00074 R<sup>2</sup>=0.969</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-A</p> <p>slope=0.00021 R<sup>2</sup>=0.921</p> <p>Next Tab Start Prev Next</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-B</p> <p>slope=0.00128 R<sup>2</sup>=0.991</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-B</p> <p>slope=0.00071 R<sup>2</sup>=0.970</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-B</p> <p>slope=0.00016 R<sup>2</sup>=0.908</p> <p>Next Tab Start Prev Next</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-C</p> <p>slope=0.00129 R<sup>2</sup>=0.998</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-C</p> <p>slope=0.00104 R<sup>2</sup>=0.981</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 40μM 200Gly-C</p> <p>slope=0.00021 R<sup>2</sup>=0.921</p> <p>Next Tab Start Prev Next</p>

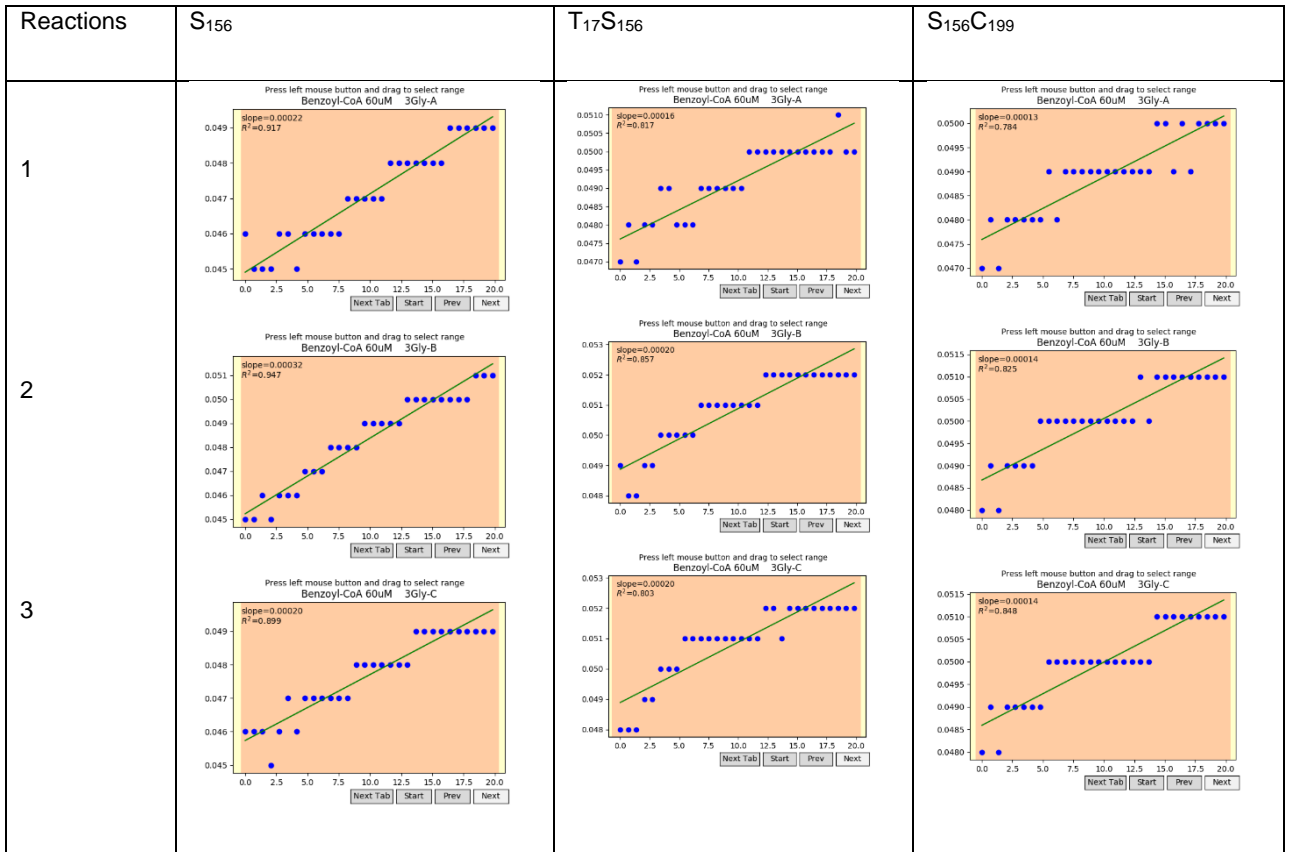
**Benzoyl-CoA : 60μM and Glycine 1mM**



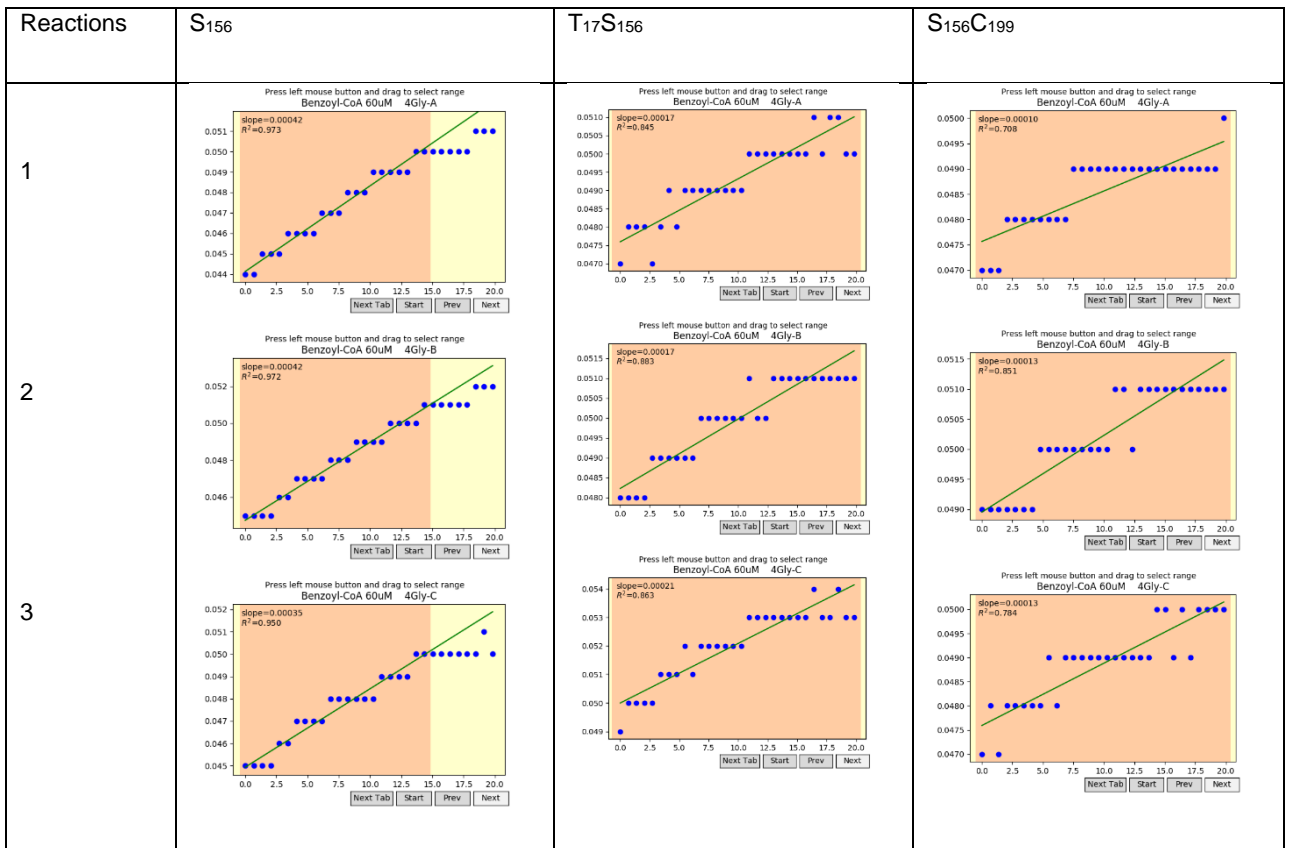
**Benzoyl-CoA : 60μM and Glycine 2mM**



### Benzoyl-CoA : 60µM and Glycine 3mM



### Benzoyl-CoA : 60µM and Glycine 4mM



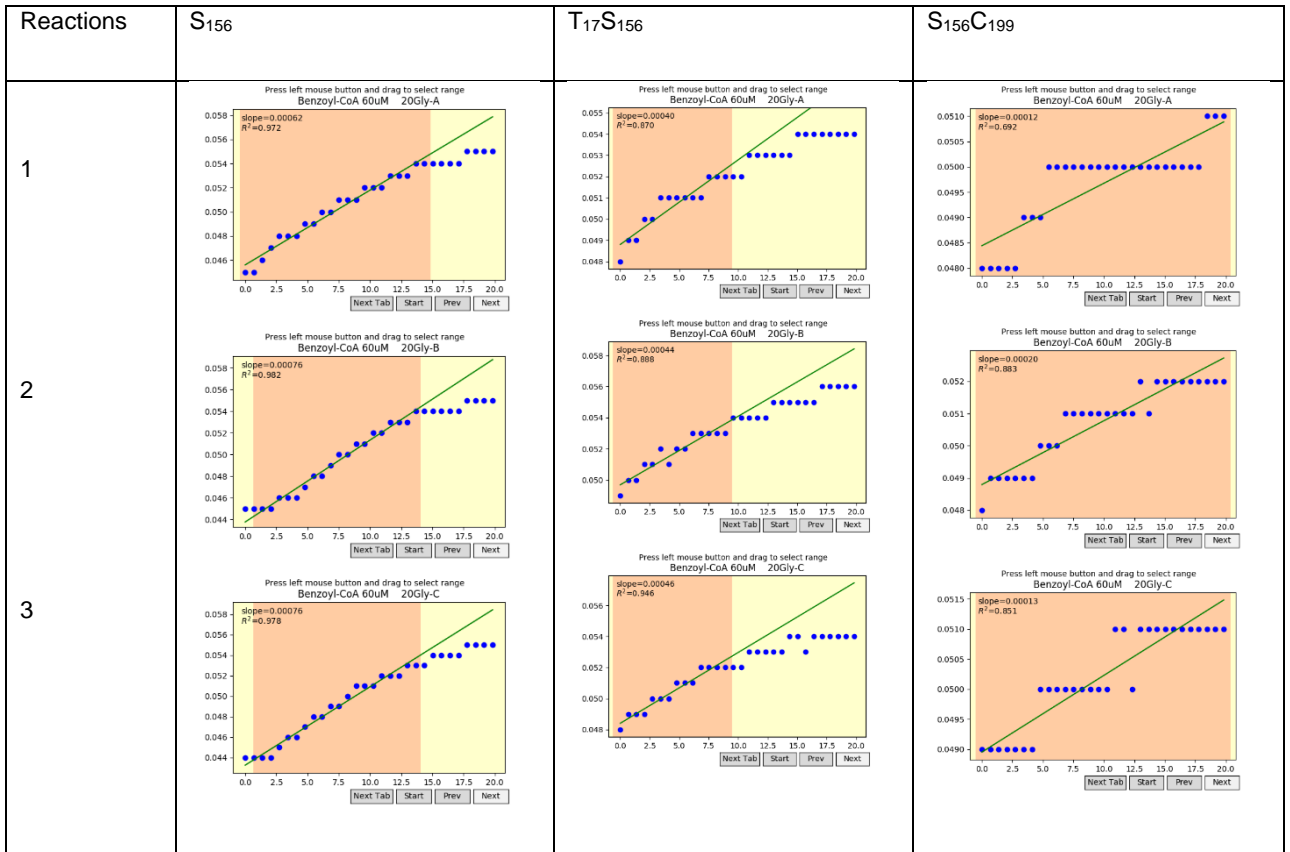
### Benzoyl-CoA : 60μM and Glycine 5mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 5Gly-C</p>

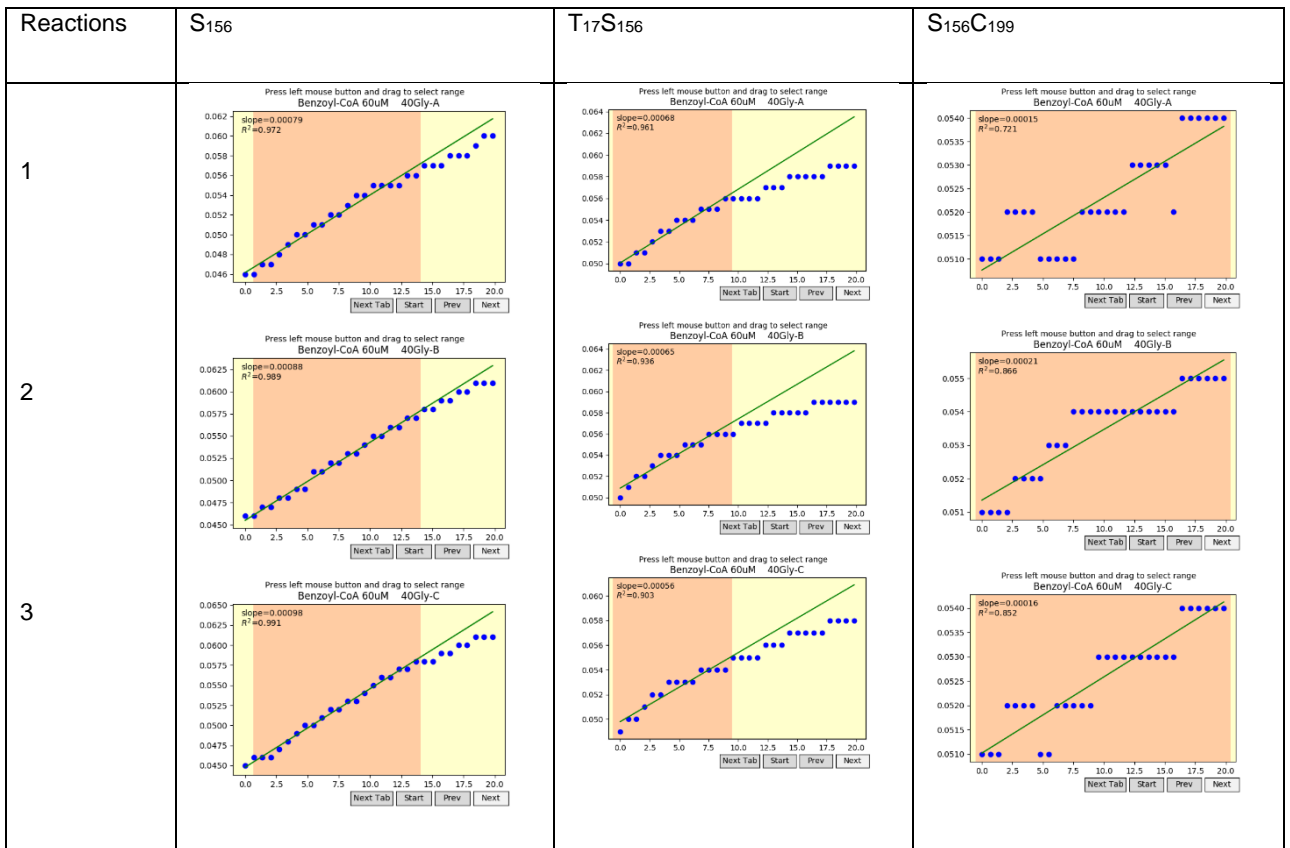
### Benzoyl-CoA : 60μM and Glycine 10mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 10Gly-C</p>

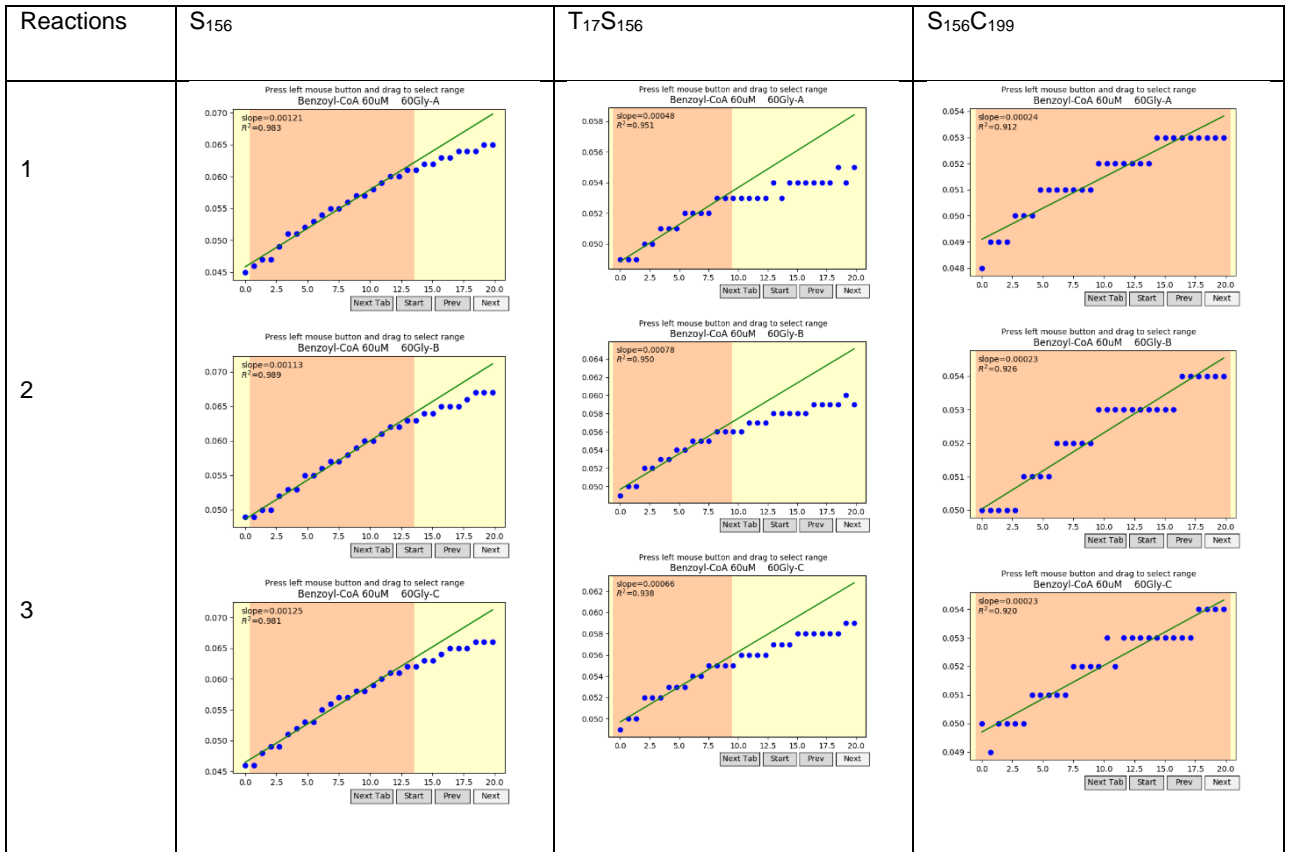
## Benzoyl-CoA : 60μM and Glycine 20mM



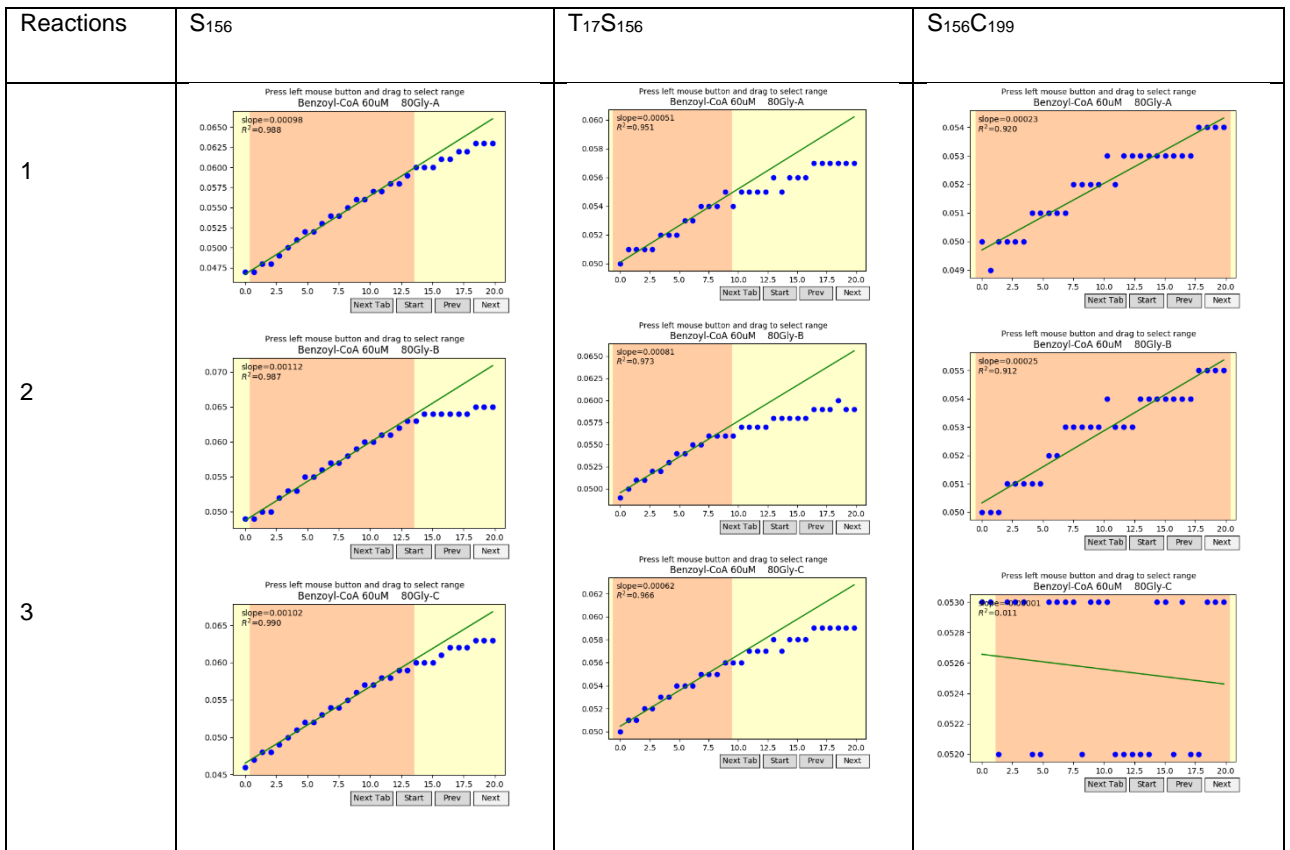
## Benzoyl-CoA : 60μM and Glycine 40mM



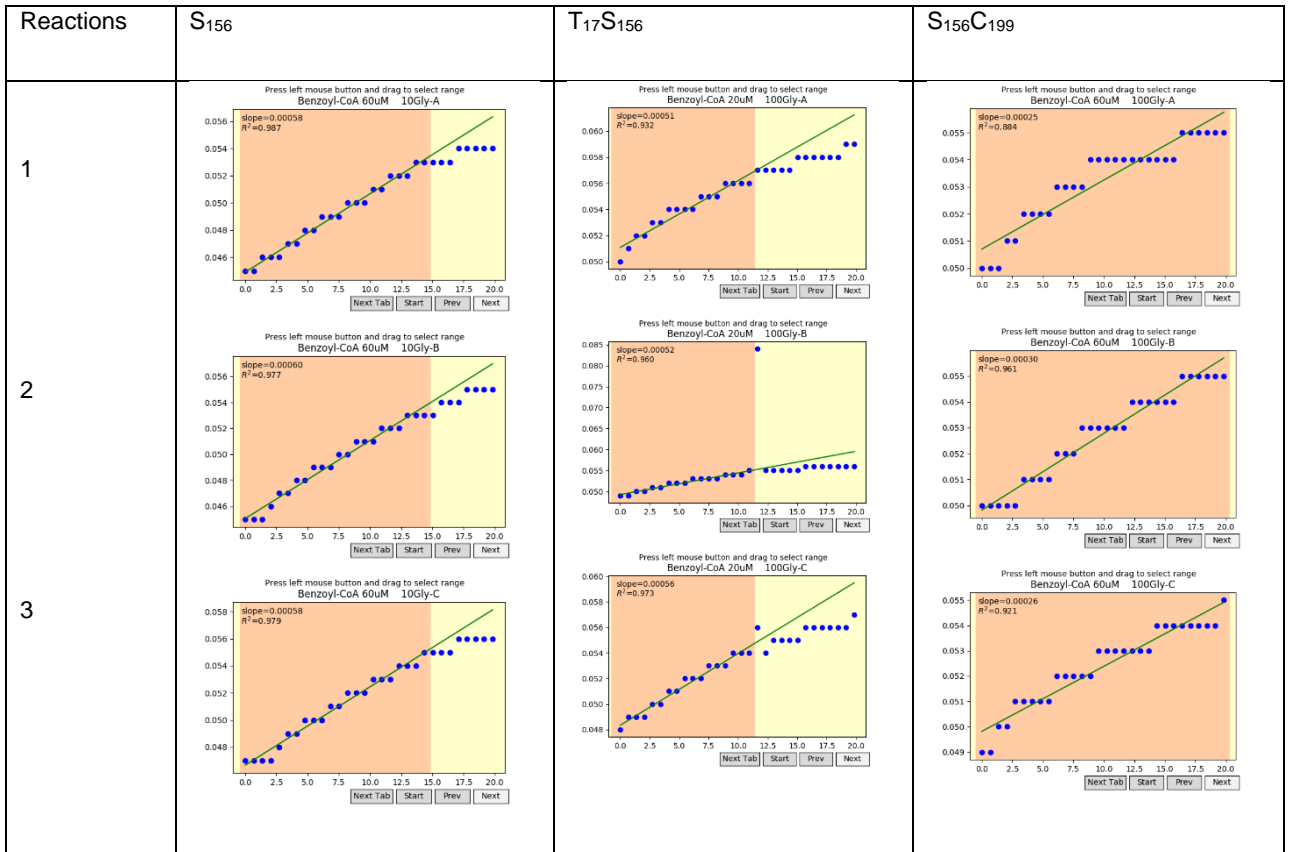
### Benzoyl-CoA : 60μM and Glycine 60mM



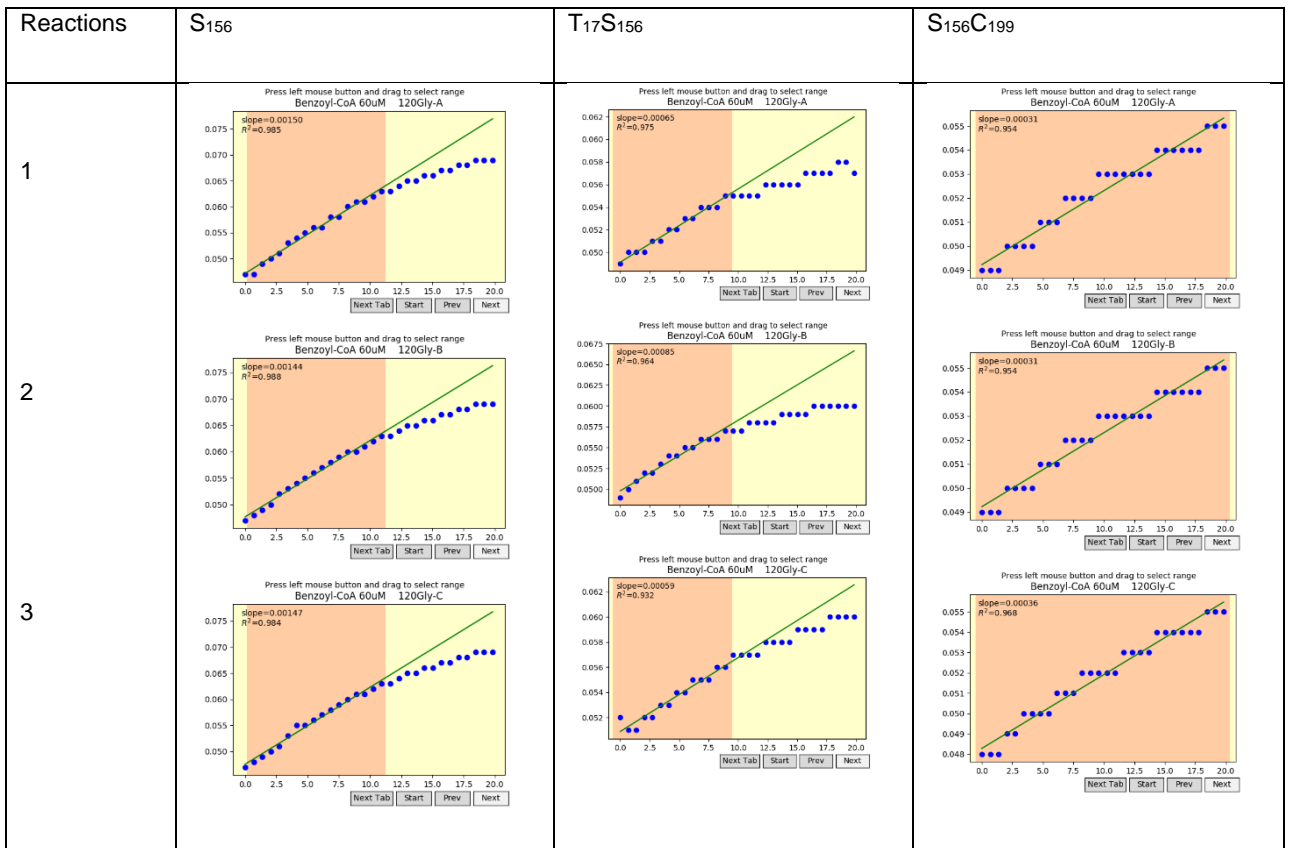
### Benzoyl-CoA : 60μM and Glycine 80mM



### Benzoyl-CoA : 60μM and Glycine 100mM



### Benzoyl-CoA : 60μM and Glycine 120mM



## Benzoyl-CoA : 60μM and Glycine 150mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-A</p> <p>slope=0.00135 R<sup>2</sup>=0.987</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-A</p> <p>slope=0.00060 R<sup>2</sup>=0.973</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-A</p> <p>slope=0.00026 R<sup>2</sup>=0.921</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-B</p> <p>slope=0.00126 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-B</p> <p>slope=0.00062 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-B</p> <p>slope=0.00023 R<sup>2</sup>=0.911</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-C</p> <p>slope=0.00134 R<sup>2</sup>=0.982</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-C</p> <p>slope=0.00076 R<sup>2</sup>=0.974</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-C</p> <p>slope=0.00028 R<sup>2</sup>=0.942</p>

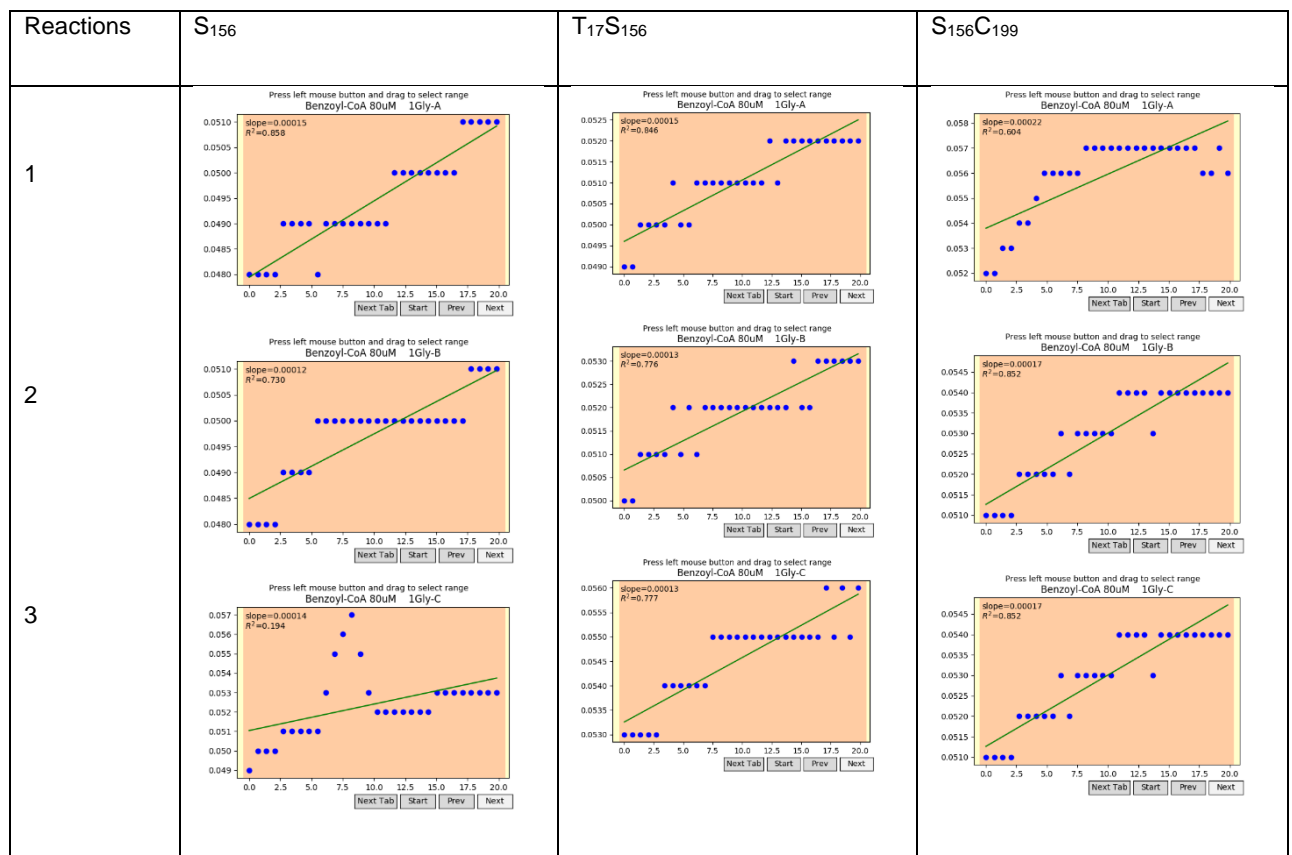
## Benzoyl-CoA : 60μM and Glycine 180mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-A</p> <p>slope=0.00122 R<sup>2</sup>=0.983</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-A</p> <p>slope=0.00063 R<sup>2</sup>=0.972</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-A</p> <p>slope=0.00029 R<sup>2</sup>=0.919</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-B</p> <p>slope=0.00084 R<sup>2</sup>=0.983</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-B</p> <p>slope=0.00062 R<sup>2</sup>=0.966</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-B</p> <p>slope=0.00027 R<sup>2</sup>=0.929</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-C</p> <p>slope=0.00109 R<sup>2</sup>=0.976</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 180Gly-C</p> <p>slope=0.00053 R<sup>2</sup>=0.958</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 150Gly-C</p> <p>slope=0.00029 R<sup>2</sup>=0.919</p>

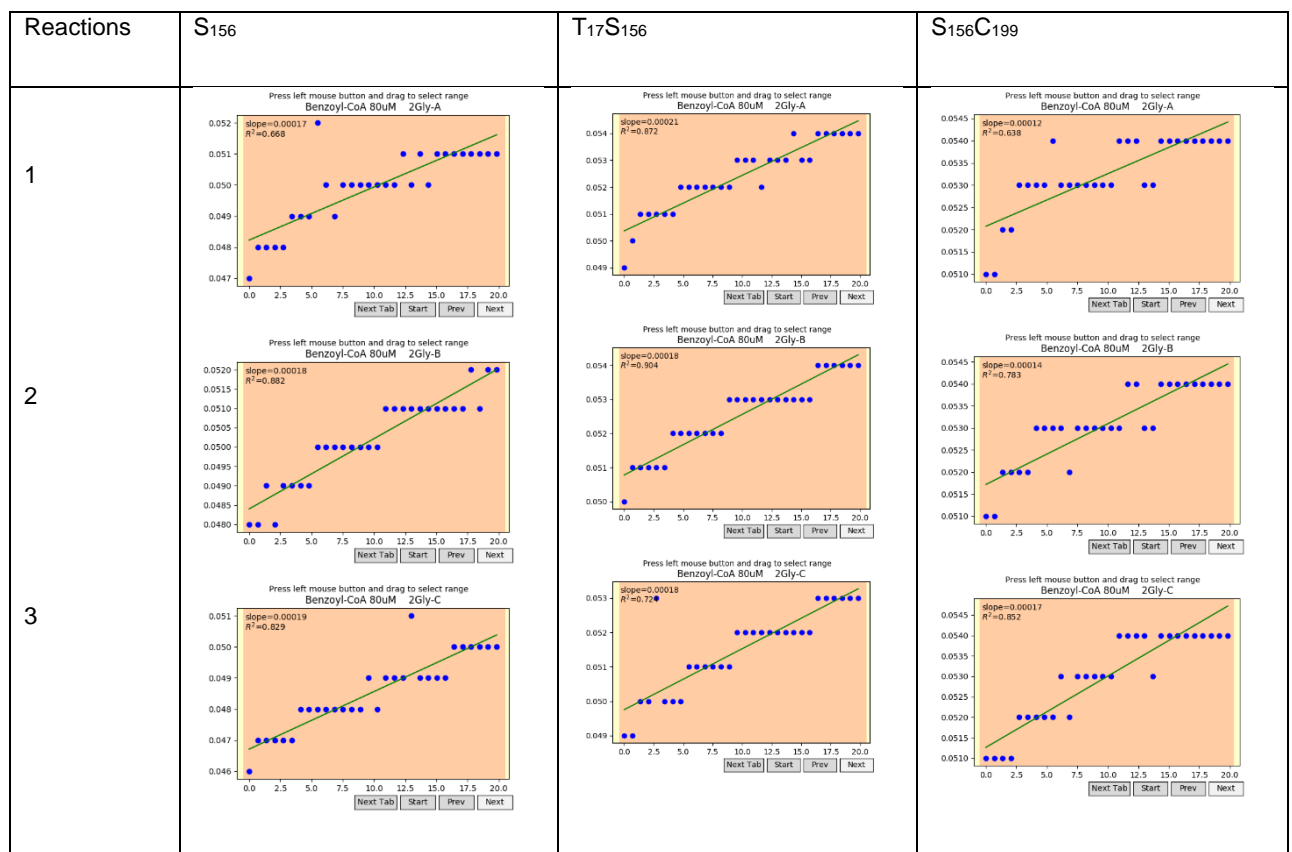
**Benzoyl-CoA : 60μM and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-A</p> <p>slope=0.00135 R<sup>2</sup>=0.989</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-A</p> <p>slope=0.00061 R<sup>2</sup>=0.945</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-A</p> <p>slope=0.00028 R<sup>2</sup>=0.942</p> <p>Next Tab Start Prev Next</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-B</p> <p>slope=0.00129 R<sup>2</sup>=0.986</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-B</p> <p>slope=0.00070 R<sup>2</sup>=0.935</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-B</p> <p>slope=0.00029 R<sup>2</sup>=0.924</p> <p>Next Tab Start Prev Next</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-C</p> <p>slope=0.00132 R<sup>2</sup>=0.996</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-C</p> <p>slope=0.00071 R<sup>2</sup>=0.967</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 60μM 200Gly-C</p> <p>slope=0.00026 R<sup>2</sup>=0.921</p> <p>Next Tab Start Prev Next</p>

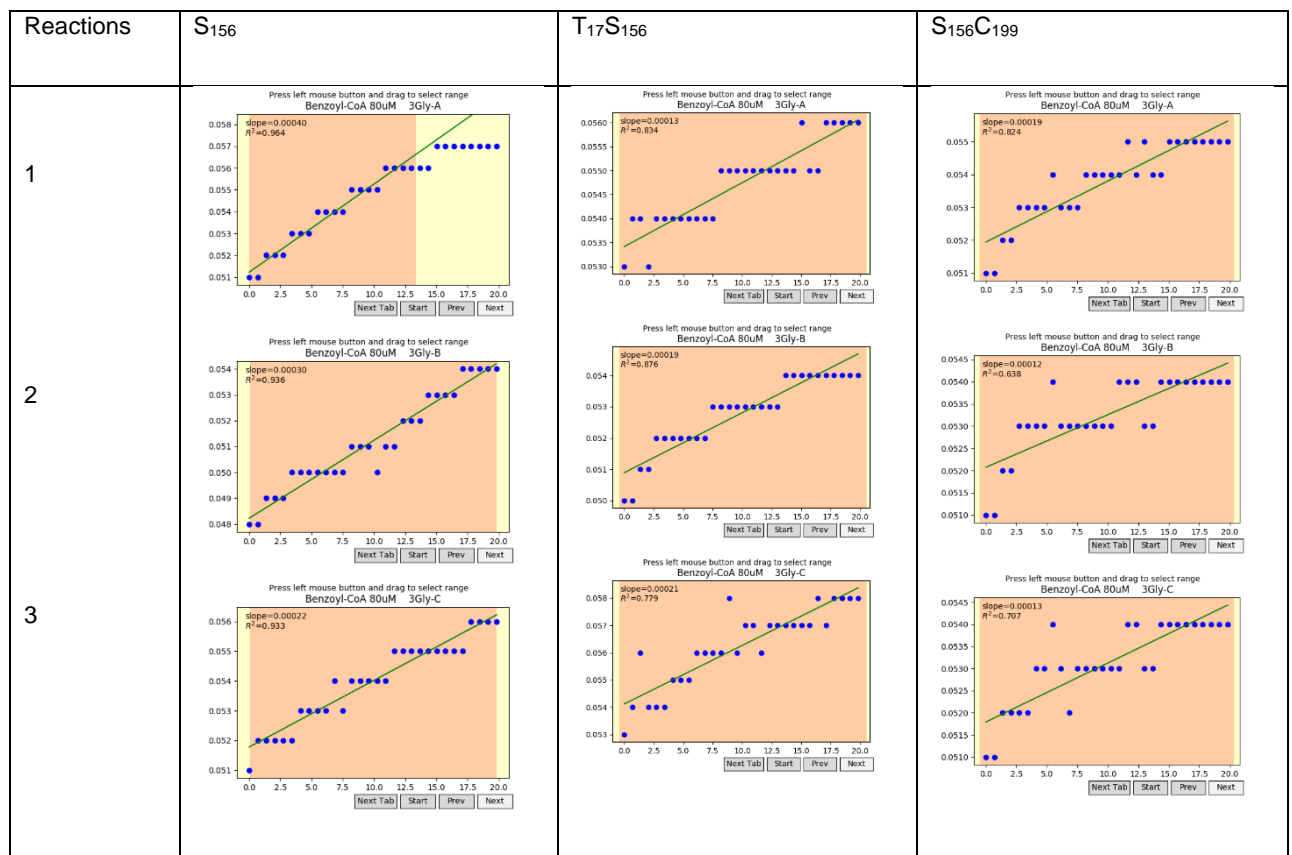
**Benzoyl-CoA : 80µM and Glycine 1mM**



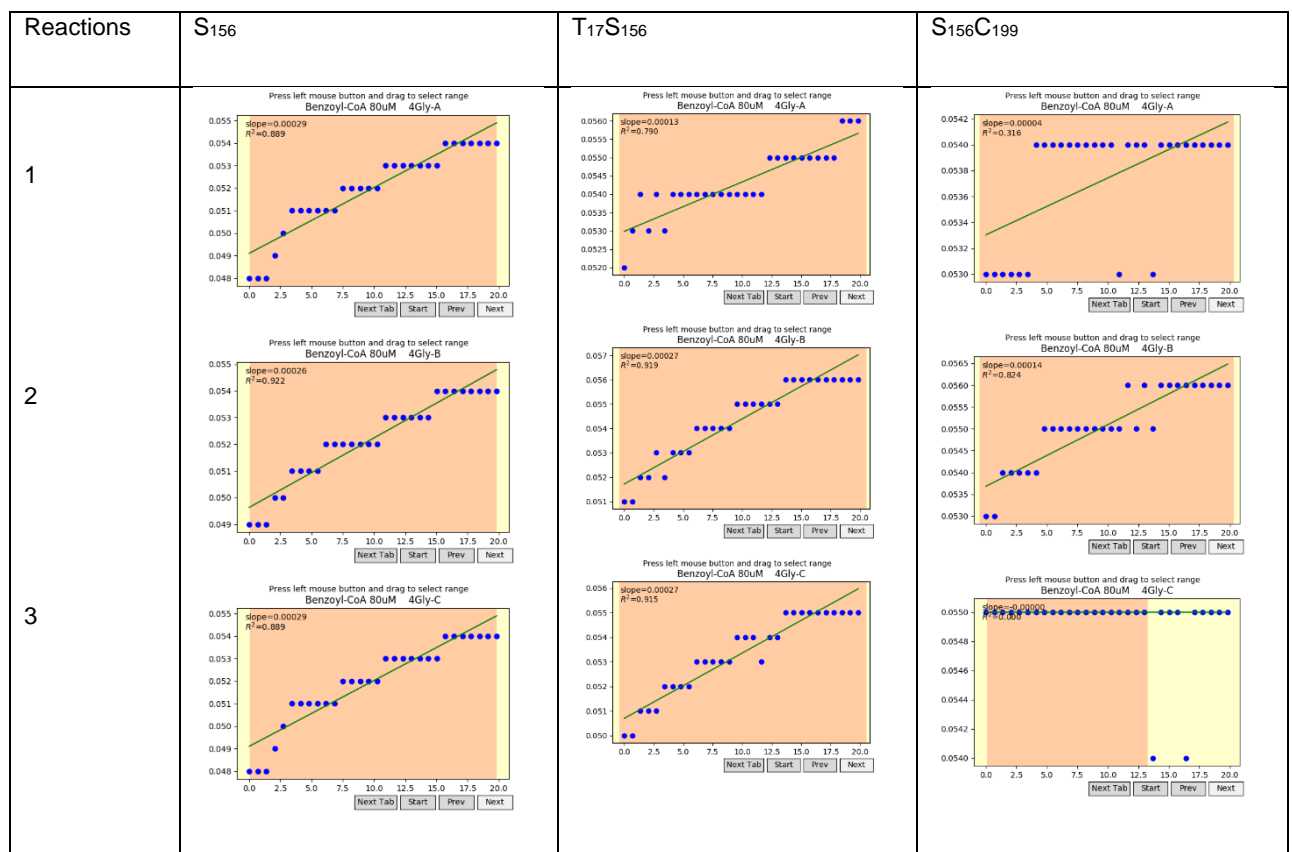
**Benzoyl-CoA : 80µM and Glycine 2mM**



### Benzoyl-CoA : 80µM and Glycine 3mM



### Benzoyl-CoA : 80µM and Glycine 4mM



**Benzoyl-CoA : 80µM and Glycine 5mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-A</p> <p>slope=0.00041 R<sup>2</sup>=0.944</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-A</p> <p>slope=0.00033 R<sup>2</sup>=0.949</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-A</p> <p>slope=0.00016 R<sup>2</sup>=0.881</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-B</p> <p>slope=0.00053 R<sup>2</sup>=0.935</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-B</p> <p>slope=0.00031 R<sup>2</sup>=0.866</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-B</p> <p>slope=0.00015 R<sup>2</sup>=0.842</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-C</p> <p>slope=0.00053 R<sup>2</sup>=0.935</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-C</p> <p>slope=0.00024 R<sup>2</sup>=0.763</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 5Gly-C</p> <p>slope=0.00017 R<sup>2</sup>=0.852</p>

**Benzoyl-CoA : 80µM and Glycine 10mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-A</p> <p>slope=0.00052 R<sup>2</sup>=0.948</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-A</p> <p>slope=0.00031 R<sup>2</sup>=0.791</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-A</p> <p>slope=0.00016 R<sup>2</sup>=0.881</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-B</p> <p>slope=0.00050 R<sup>2</sup>=0.955</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-B</p> <p>slope=0.00028 R<sup>2</sup>=0.744</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-B</p> <p>slope=0.00014 R<sup>2</sup>=0.848</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-C</p> <p>slope=0.00051 R<sup>2</sup>=0.929</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-C</p> <p>slope=0.00043 R<sup>2</sup>=0.820</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 10Gly-C</p> <p>slope=0.00021 R<sup>2</sup>=0.858</p>

### Benzoyl-CoA : 80µM and Glycine 20mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-A</p> <p>slope=0.00075 R<sup>2</sup>=0.974</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-A</p> <p>slope=0.00088 R<sup>2</sup>=0.972</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-A</p> <p>slope=0.00017 R<sup>2</sup>=0.589</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-B</p> <p>slope=0.00081 R<sup>2</sup>=0.969</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-B</p> <p>slope=0.00067 R<sup>2</sup>=0.962</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-B</p> <p>slope=0.00019 R<sup>2</sup>=0.835</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-C</p> <p>slope=0.00077 R<sup>2</sup>=0.973</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-C</p> <p>slope=0.00089 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 20Gly-C</p> <p>slope=0.00018 R<sup>2</sup>=0.707</p>

### Benzoyl-CoA : 80µM and Glycine 40mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-A</p> <p>slope=0.00101 R<sup>2</sup>=0.988</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-A</p> <p>slope=0.00101 R<sup>2</sup>=0.981</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-A</p> <p>slope=0.00030 R<sup>2</sup>=0.828</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-B</p> <p>slope=0.00108 R<sup>2</sup>=0.988</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-B</p> <p>slope=0.00094 R<sup>2</sup>=0.921</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-B</p> <p>slope=0.00031 R<sup>2</sup>=0.905</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-C</p> <p>slope=0.00119 R<sup>2</sup>=0.978</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-C</p> <p>slope=0.00116 R<sup>2</sup>=0.990</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80µM 40Gly-C</p> <p>slope=0.00034 R<sup>2</sup>=0.899</p>

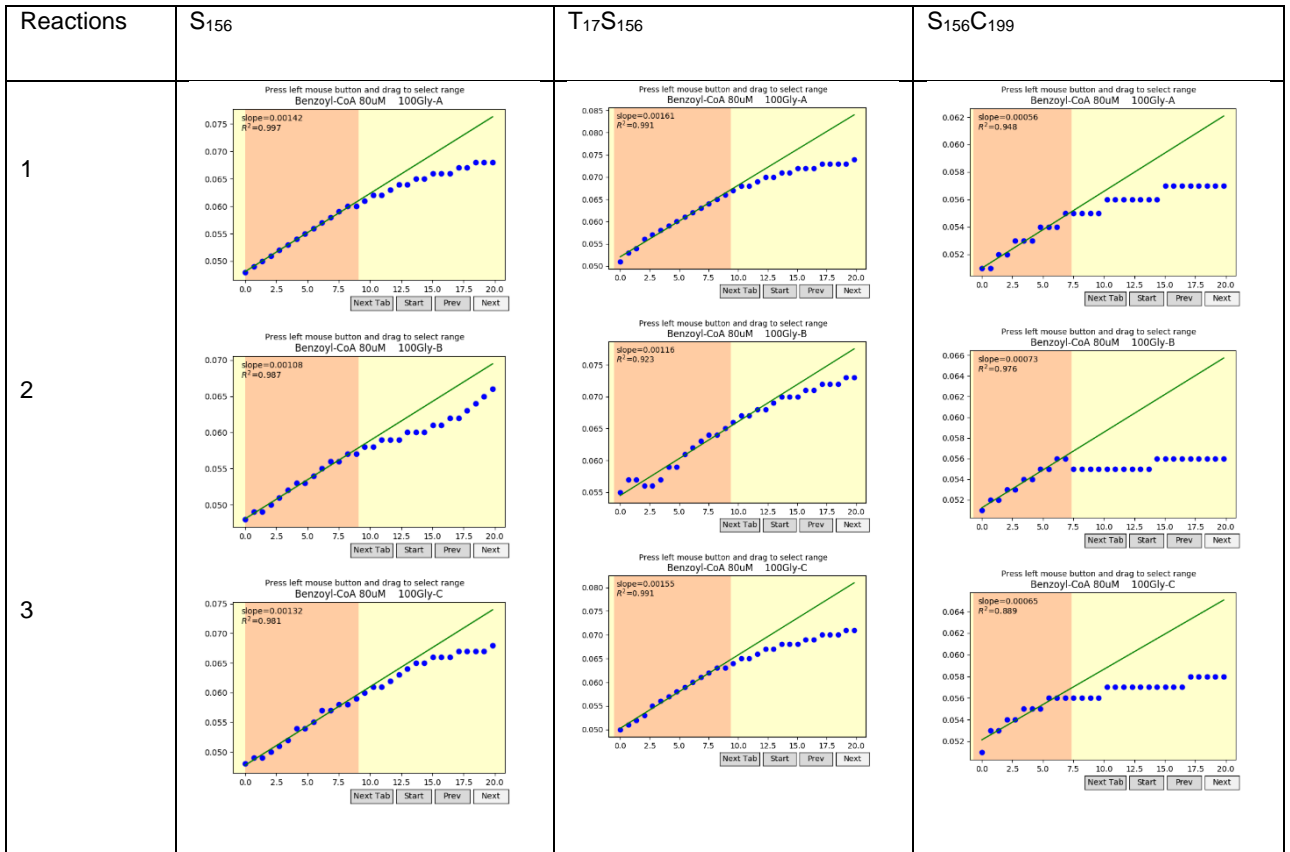
### Benzoyl-CoA : 80μM and Glycine 60mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-A</p> <p>slope=0.00116 R<sup>2</sup>=0.983</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-A</p> <p>slope=0.00158 R<sup>2</sup>=0.993</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-A</p> <p>slope=0.00033 R<sup>2</sup>=0.895</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-B</p> <p>slope=0.00134 R<sup>2</sup>=0.980</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-B</p> <p>slope=0.00115 R<sup>2</sup>=0.981</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-B</p> <p>slope=0.00037 R<sup>2</sup>=0.835</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-C</p> <p>slope=0.00136 R<sup>2</sup>=0.990</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-C</p> <p>slope=0.00137 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 60Gly-C</p> <p>slope=0.00033 R<sup>2</sup>=0.895</p>

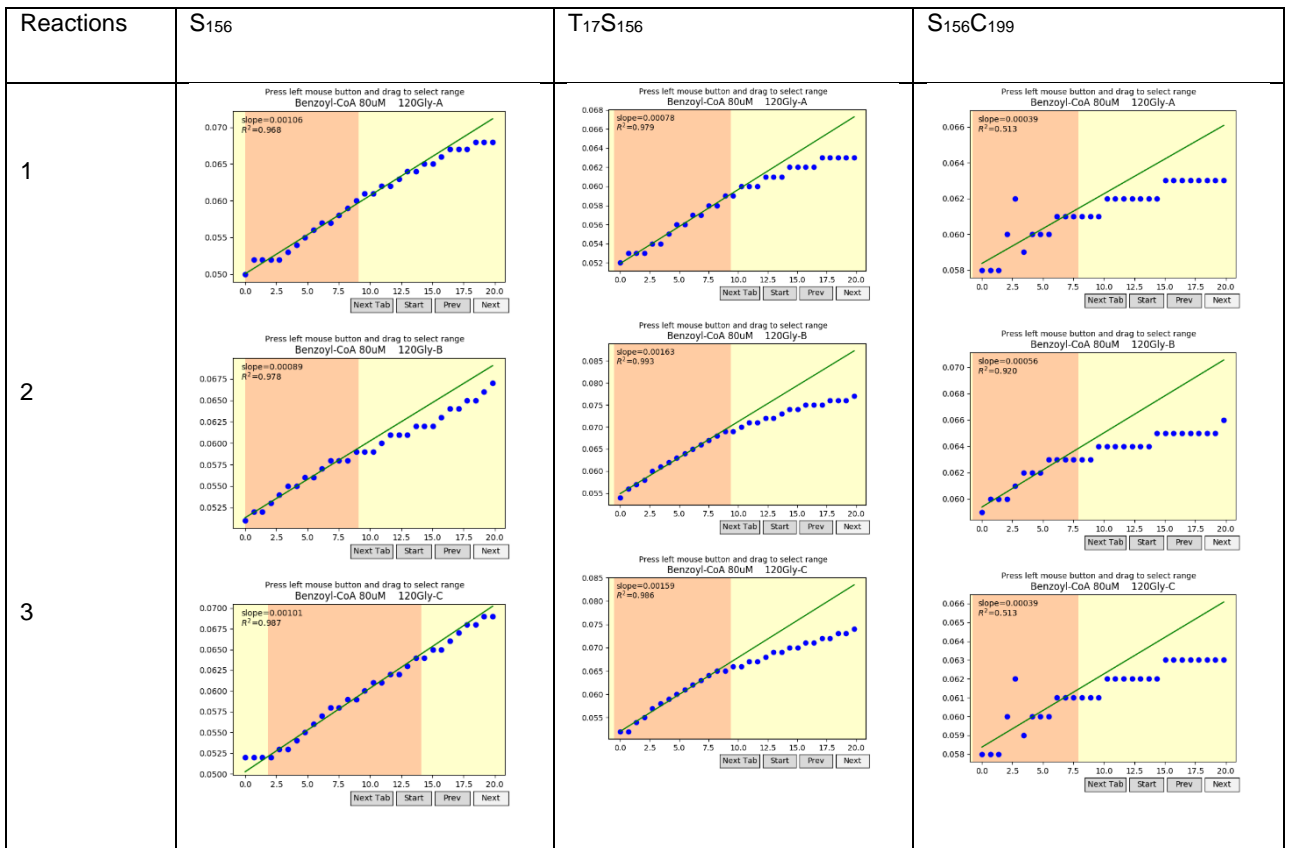
### Benzoyl-CoA : 80μM and Glycine 80mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-A</p> <p>slope=0.00139 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-A</p> <p>slope=0.00141 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-A</p> <p>slope=0.00029 R<sup>2</sup>=0.823</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-B</p> <p>slope=0.00151 R<sup>2</sup>=0.980</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-B</p> <p>slope=0.00149 R<sup>2</sup>=0.987</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-B</p> <p>slope=0.00034 R<sup>2</sup>=0.899</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-C</p> <p>slope=0.00144 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-C</p> <p>slope=0.00194 R<sup>2</sup>=0.983</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 80Gly-C</p> <p>slope=0.00037 R<sup>2</sup>=0.835</p>

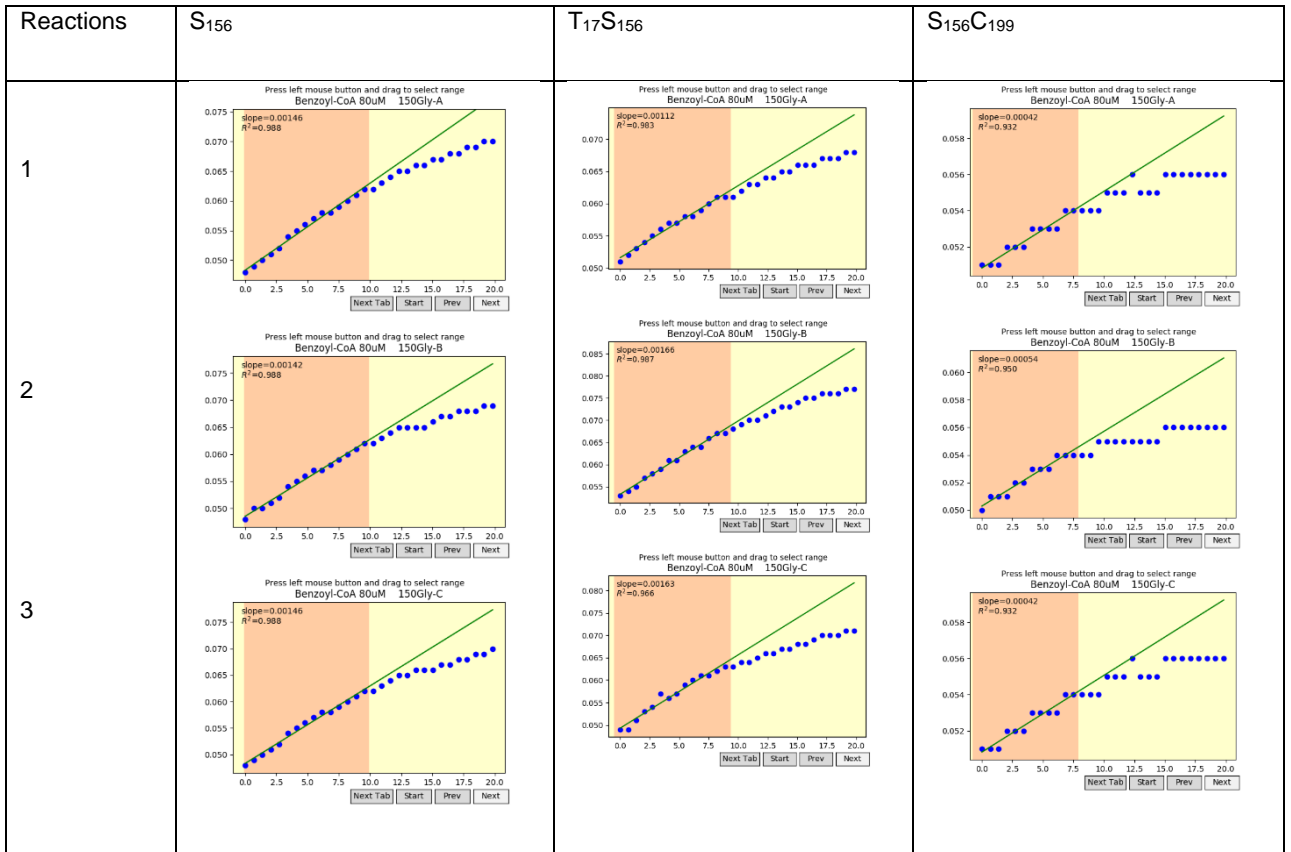
## Benzoyl-CoA : 80μM and Glycine 100mM



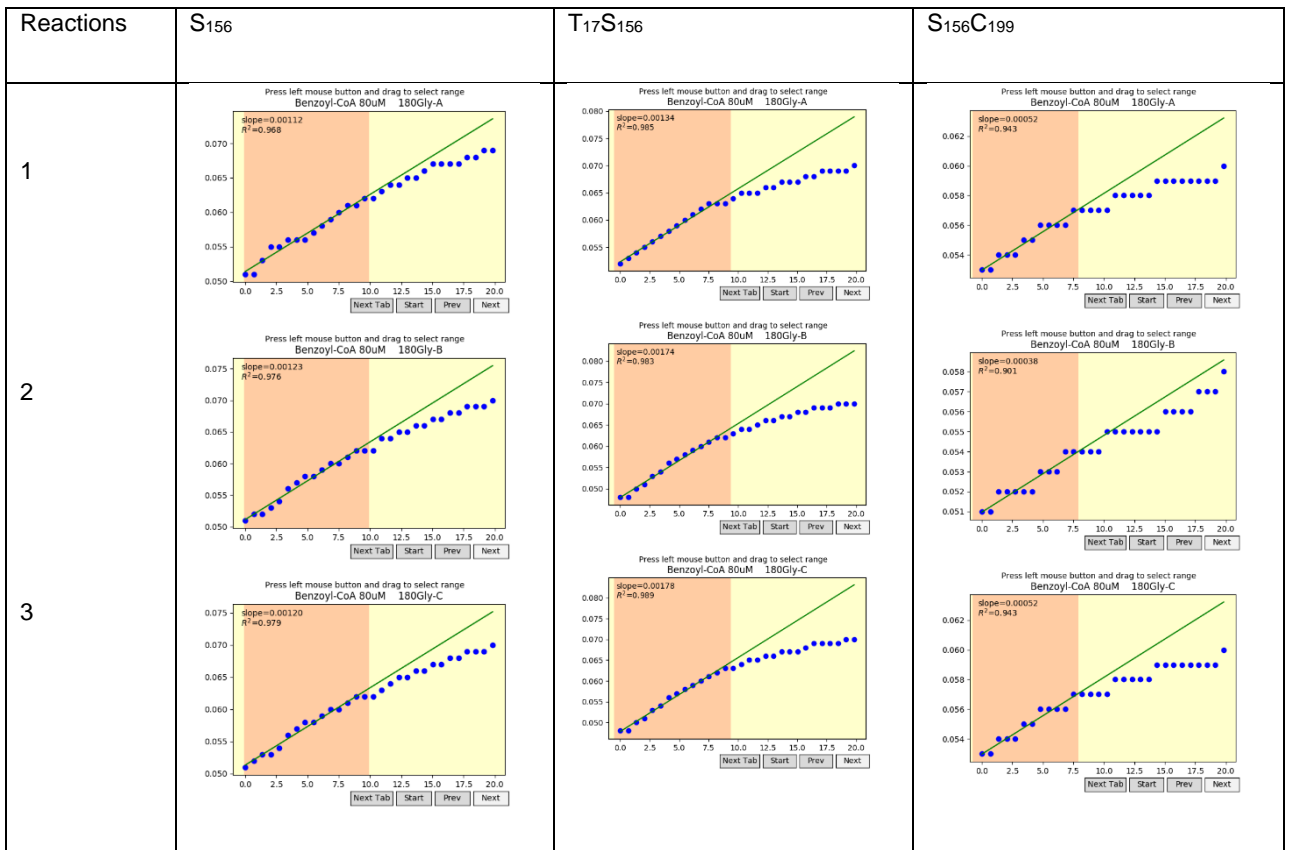
## Benzoyl-CoA : 80μM and Glycine 120mM



**Benzoyl-CoA : 80µM and Glycine 150mM**



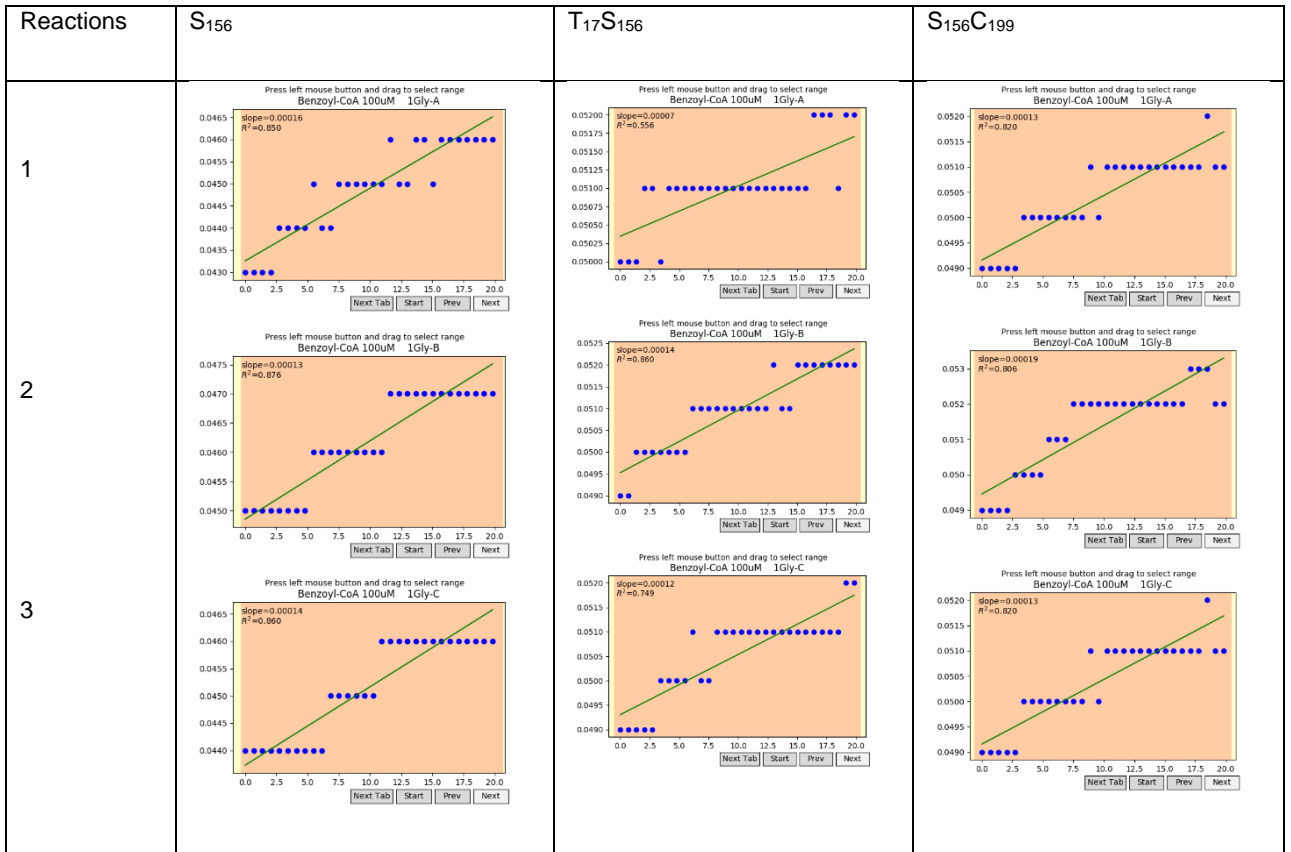
**Benzoyl-CoA : 80µM and Glycine 180mM**



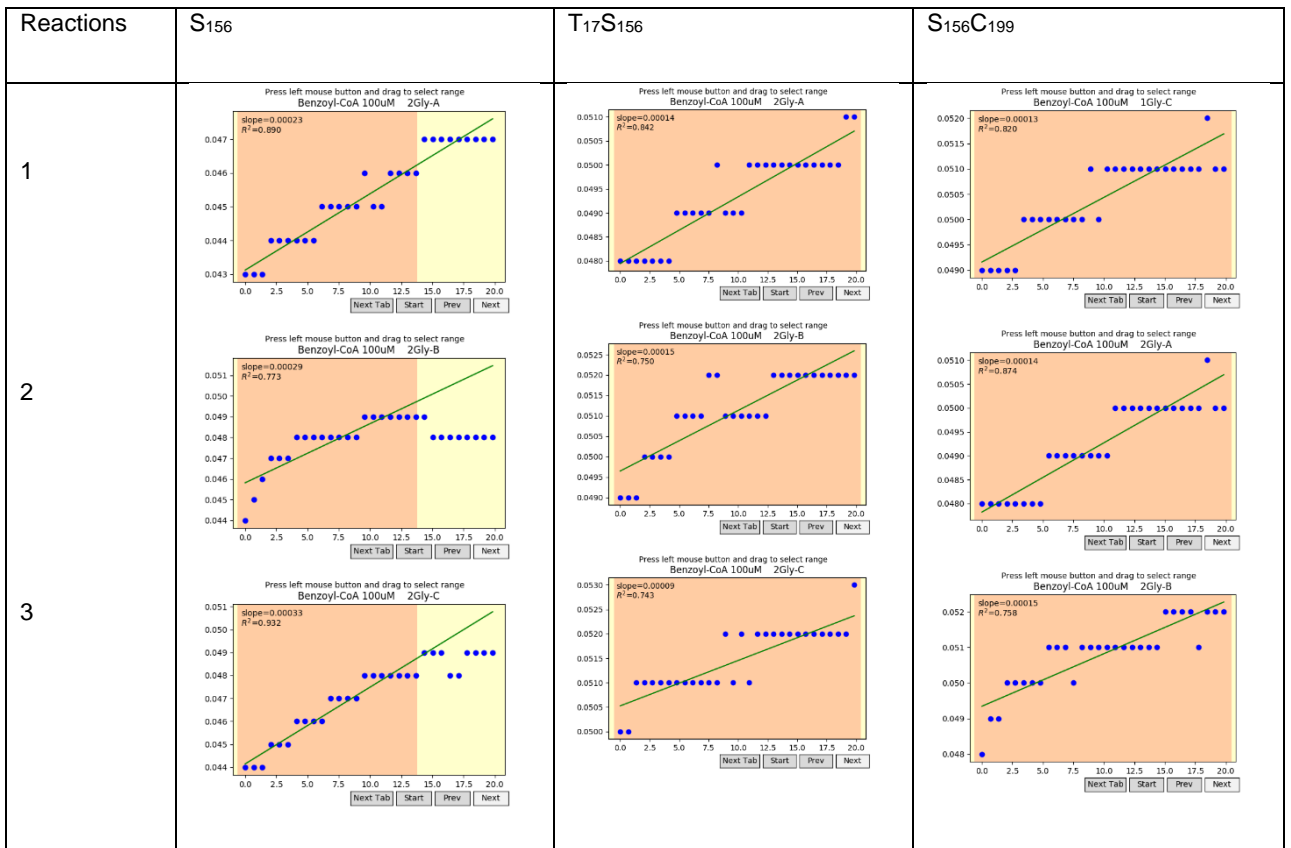
**Benzoyl-CoA : 80μM and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-A</p> <p>slope=0.00150 R<sup>2</sup>=0.997</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-A</p> <p>slope=0.00119 R<sup>2</sup>=0.990</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-A</p> <p>slope=0.00049 R<sup>2</sup>=0.933</p> <p>Next Tab Start Prev Next</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-B</p> <p>slope=0.00140 R<sup>2</sup>=0.991</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-B</p> <p>slope=0.00152 R<sup>2</sup>=0.990</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-B</p> <p>slope=0.00055 R<sup>2</sup>=0.941</p> <p>Next Tab Start Prev Next</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-C</p> <p>slope=0.00141 R<sup>2</sup>=0.987</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-C</p> <p>slope=0.00179 R<sup>2</sup>=0.986</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 80μM 200Gly-C</p> <p>slope=0.00059 R<sup>2</sup>=0.871</p> <p>Next Tab Start Prev Next</p>

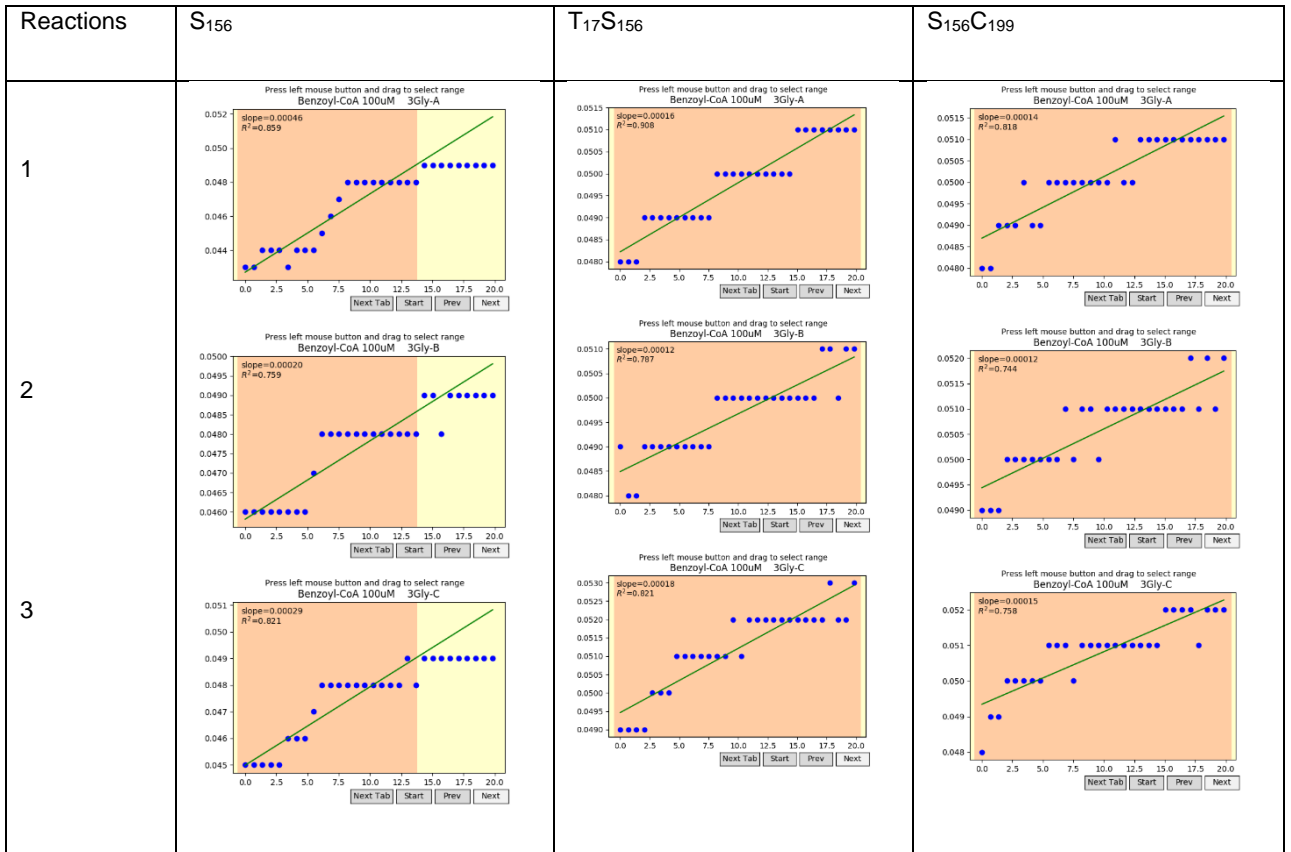
## Benzoyl-CoA : 100µM and Glycine 1mM



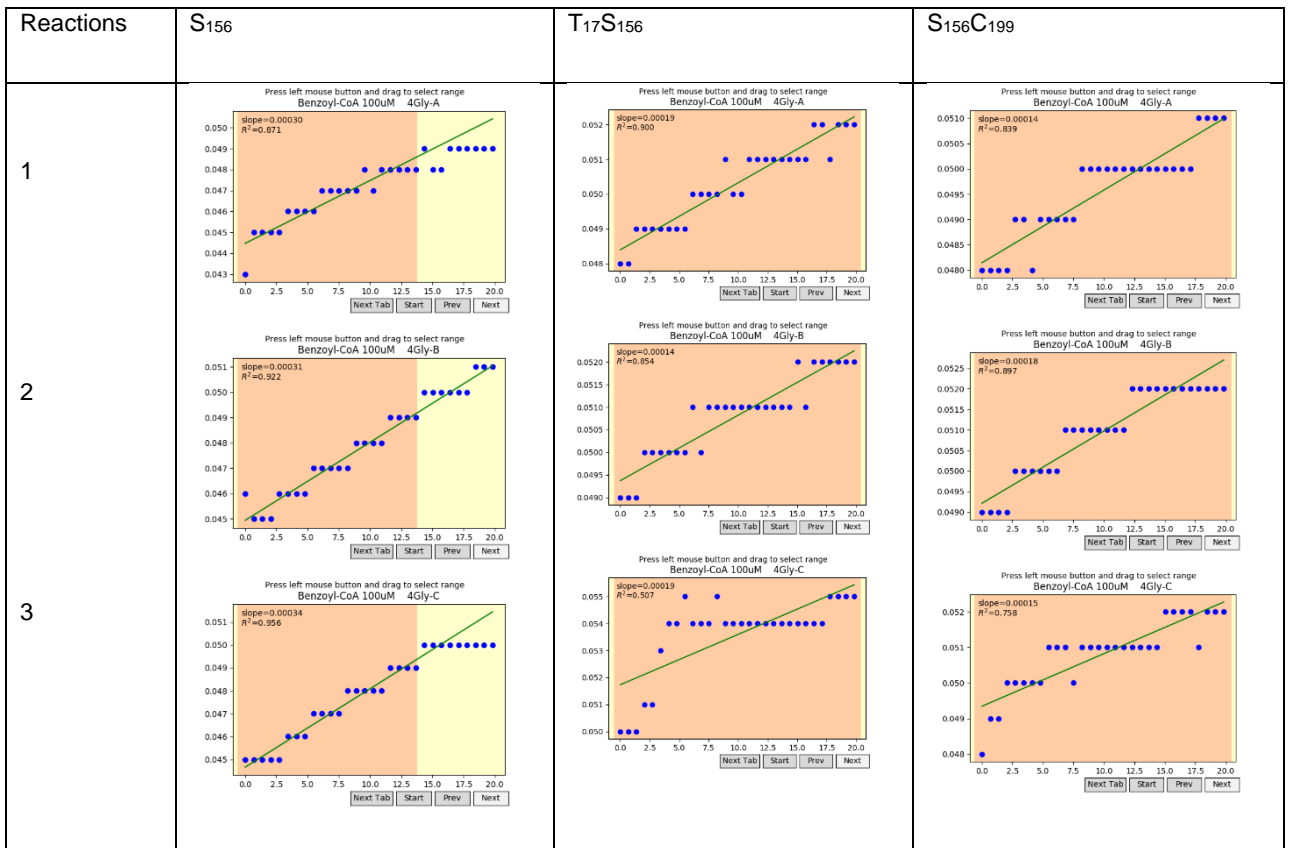
## Benzoyl-CoA : 100µM and Glycine 2mM



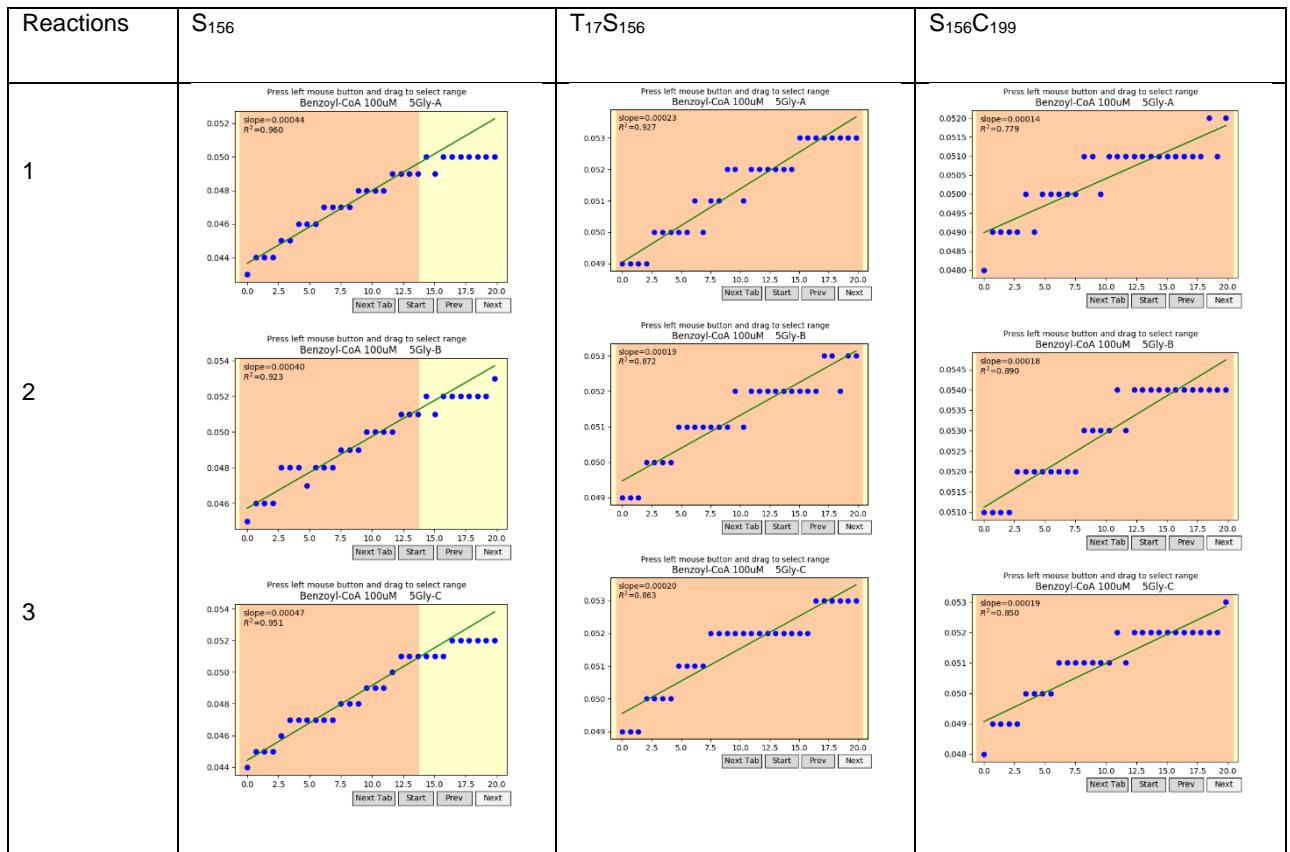
## Benzoyl-CoA : 100μM and Glycine 3mM



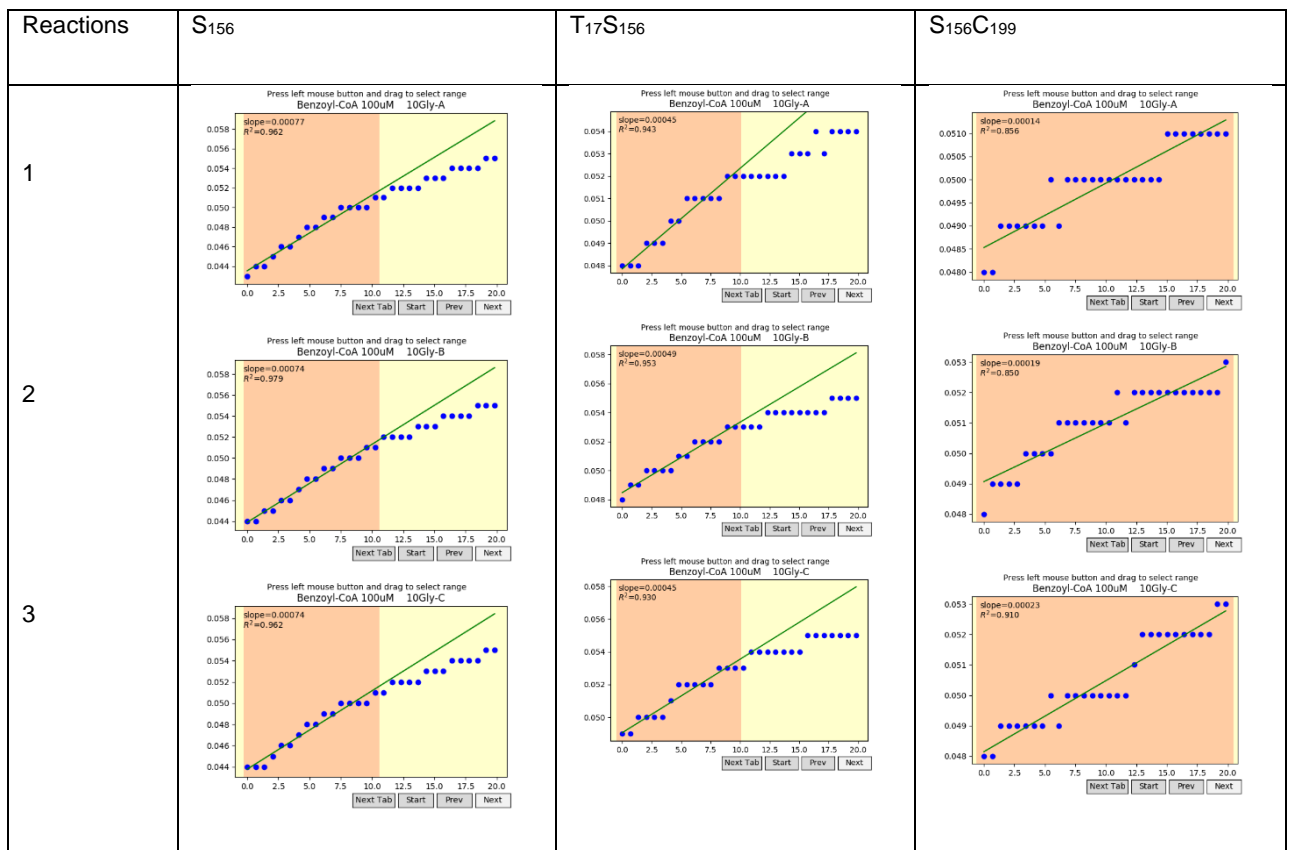
## Benzoyl-CoA : 100μM and Glycine 4mM



## Benzoyl-CoA : 100µM and Glycine 5mM



## Benzoyl-CoA : 100µM and Glycine 10mM



**Benzoyl-CoA : 100µM and Glycine 20mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-A</p> <p>slope=0.00119 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-A</p> <p>slope=0.00052 R<sup>2</sup>=0.957</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-A</p> <p>slope=0.00017 R<sup>2</sup>=0.841</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-B</p> <p>slope=0.00093 R<sup>2</sup>=0.893</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-B</p> <p>slope=0.00062 R<sup>2</sup>=0.955</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-B</p> <p>slope=0.00024 R<sup>2</sup>=0.878</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-C</p> <p>slope=0.00107 R<sup>2</sup>=0.972</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-C</p> <p>slope=0.00063 R<sup>2</sup>=0.973</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 20Gly-C</p> <p>slope=0.00017 R<sup>2</sup>=0.841</p>

**Benzoyl-CoA : 100µM and Glycine 40mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-A</p> <p>slope=0.00066 R<sup>2</sup>=0.969</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-A</p> <p>slope=0.00091 R<sup>2</sup>=0.975</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-A</p> <p>slope=0.00034 R<sup>2</sup>=0.823</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-B</p> <p>slope=0.00041 R<sup>2</sup>=0.948</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-B</p> <p>slope=0.00054 R<sup>2</sup>=0.929</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-B</p> <p>slope=0.00024 R<sup>2</sup>=0.789</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-C</p> <p>slope=0.00058 R<sup>2</sup>=0.961</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-C</p> <p>slope=0.00082 R<sup>2</sup>=0.957</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100µM 40Gly-C</p> <p>slope=0.00037 R<sup>2</sup>=0.918</p>

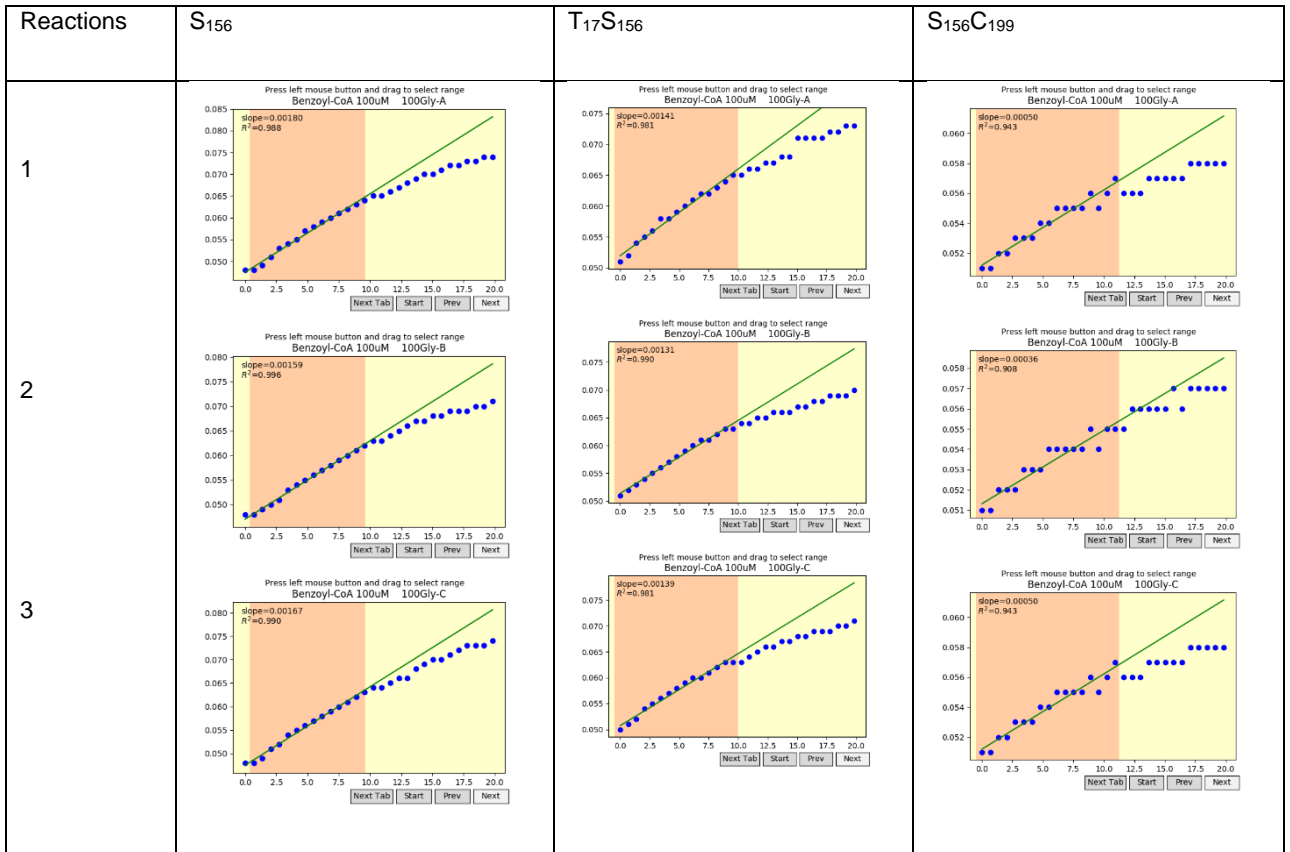
**Benzoyl-CoA : 100μM and Glycine 60mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 60Gly-C</p>

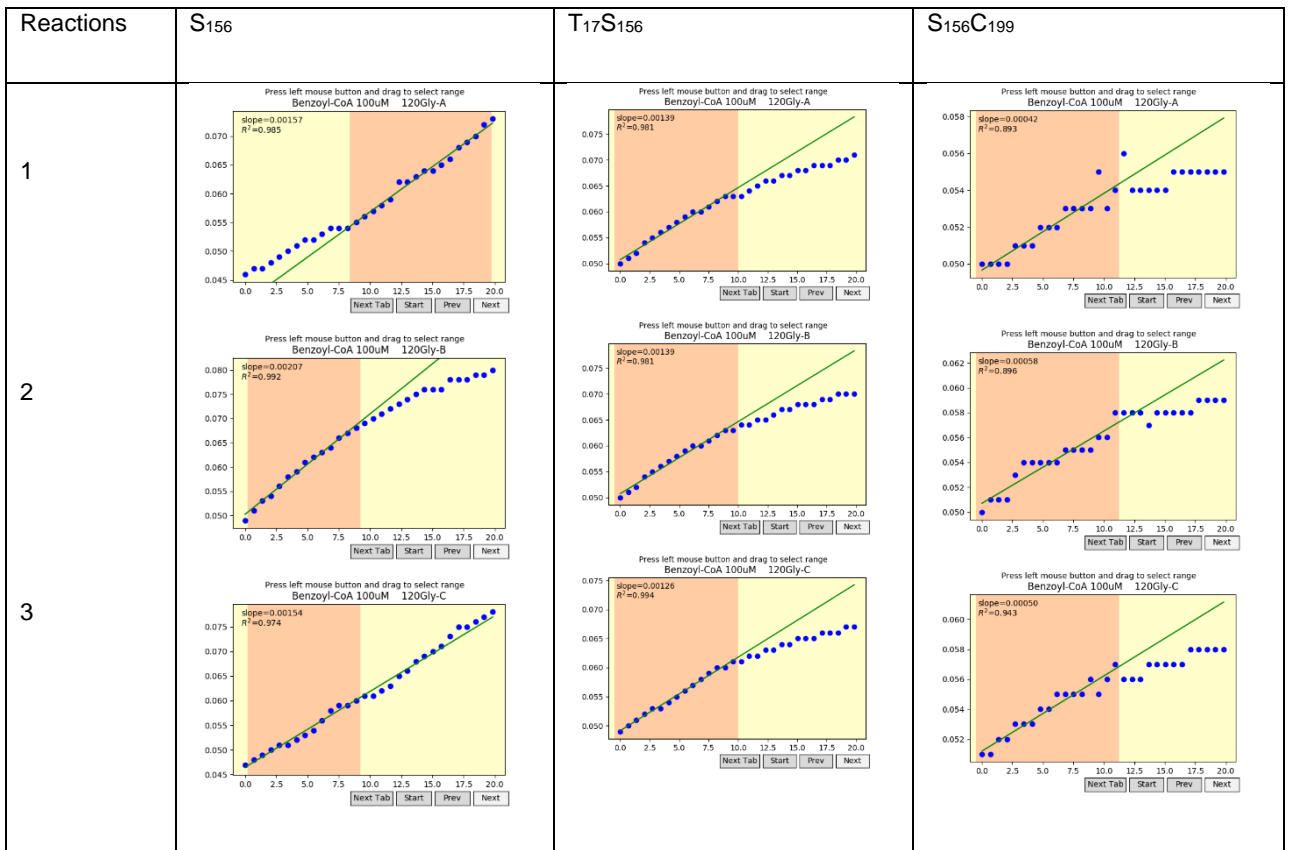
**Benzoyl-CoA : 100μM and Glycine 80mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 80Gly-C</p>

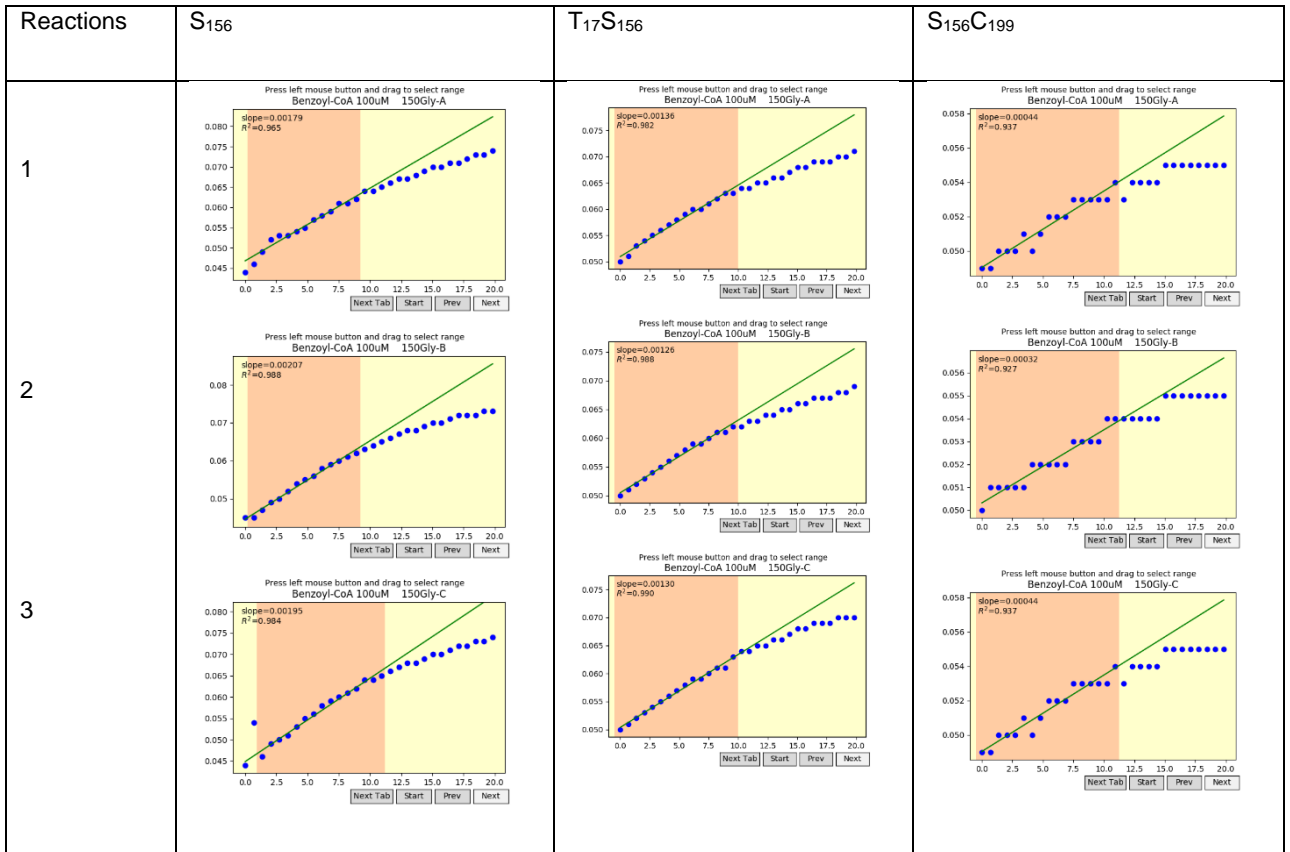
**Benzoyl-CoA : 100µM and Glycine 100mM**



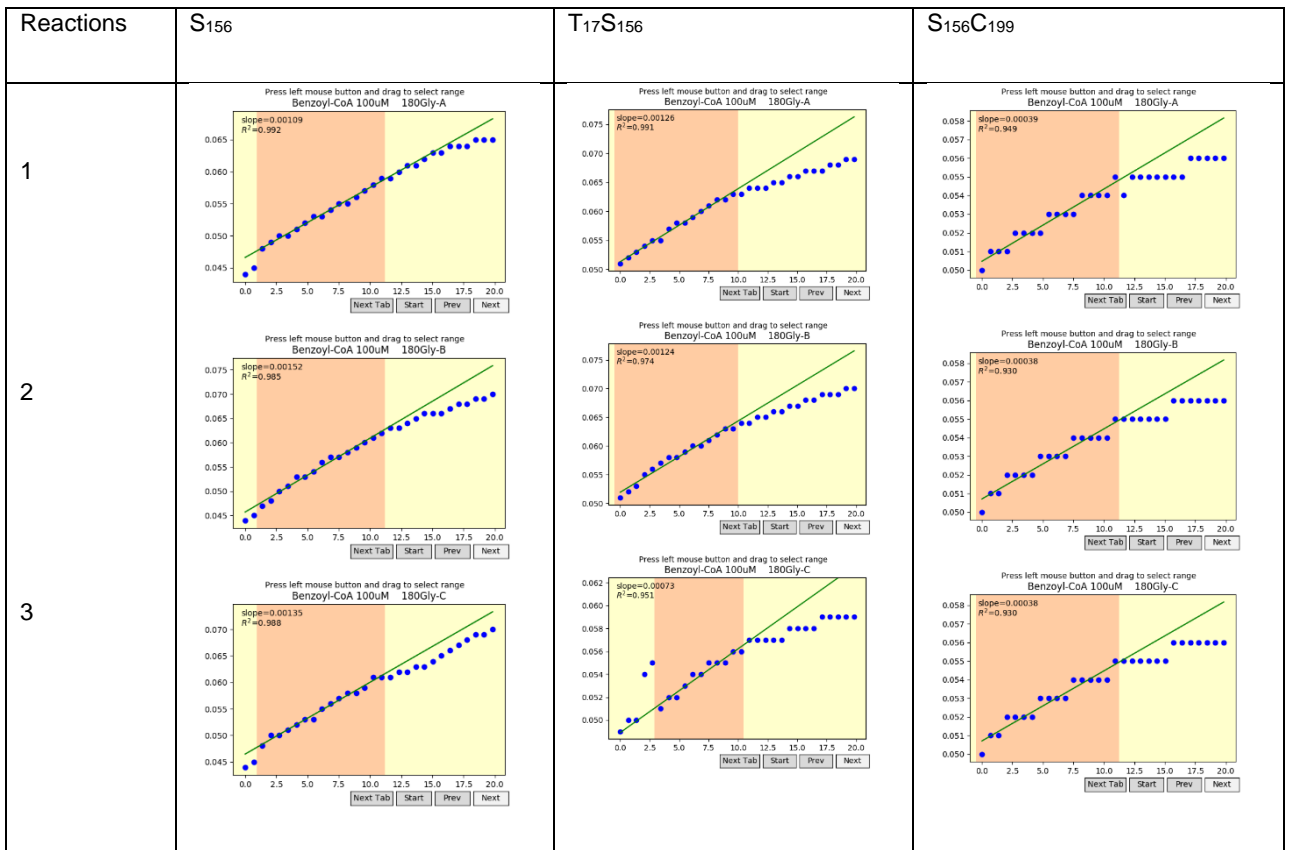
**Benzoyl-CoA : 100µM and Glycine 120mM**



**Benzoyl-CoA : 100μM and Glycine 150mM**



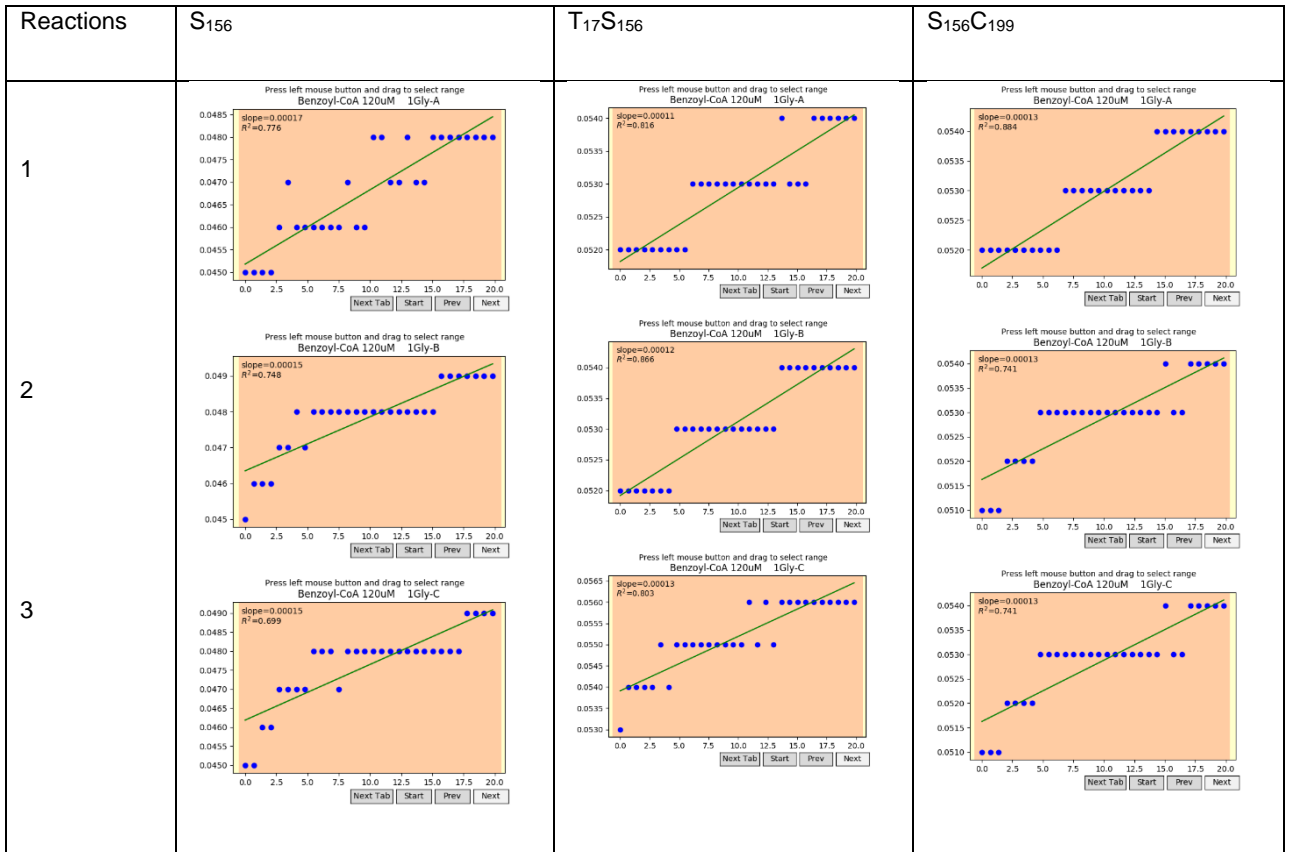
**Benzoyl-CoA : 100μM and Glycine 180mM**



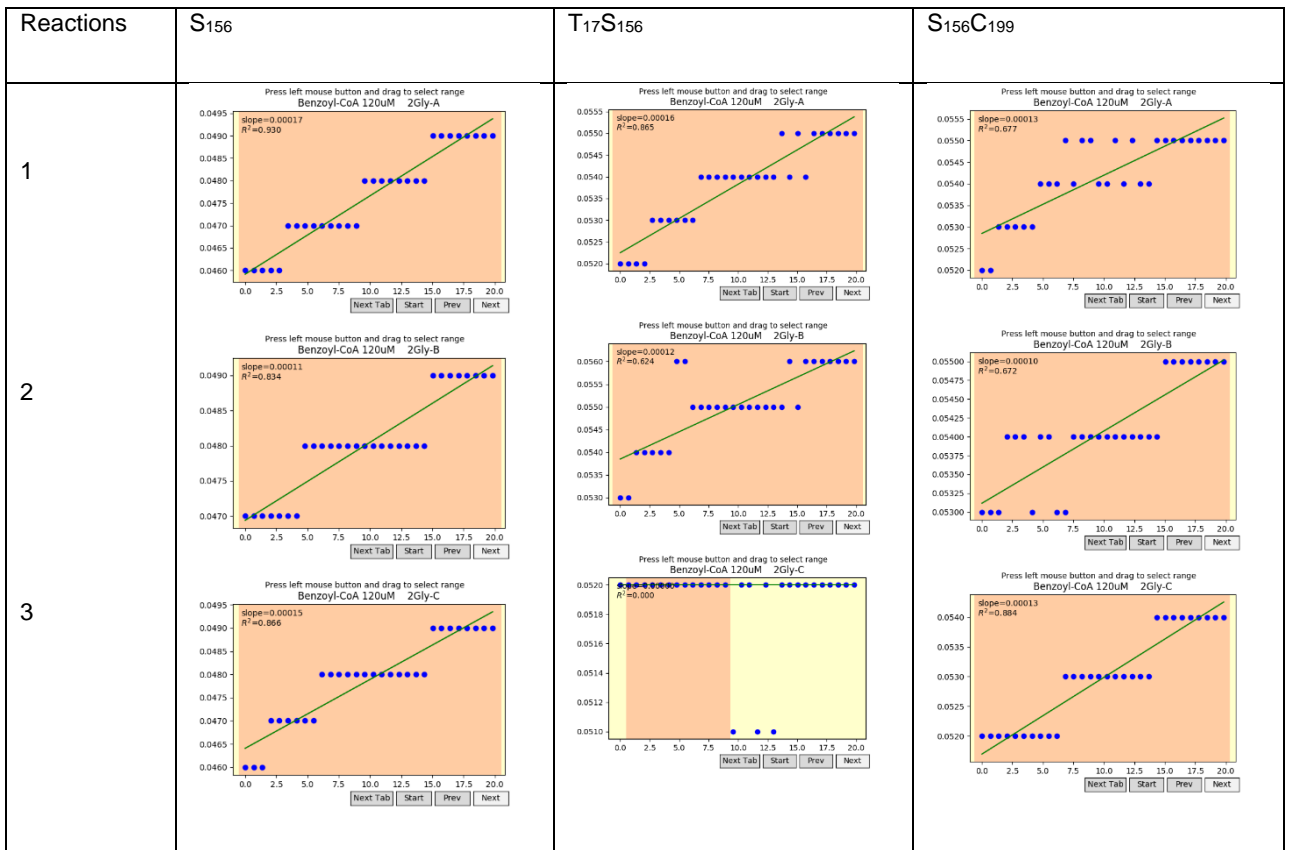
**Benzoyl-CoA : 100μM and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-A</p> <p>slope=0.00159 R<sup>2</sup>=0.992</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-A</p> <p>slope=0.00112 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-A</p> <p>slope=0.00036 R<sup>2</sup>=0.906</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-B</p> <p>slope=0.00095 R<sup>2</sup>=0.975</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-B</p> <p>slope=0.00113 R<sup>2</sup>=0.991</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-B</p> <p>slope=0.00042 R<sup>2</sup>=0.908</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-C</p> <p>slope=0.00128 R<sup>2</sup>=0.989</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-C</p> <p>slope=0.00102 R<sup>2</sup>=0.986</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 100uM 200Gly-C</p> <p>slope=0.00036 R<sup>2</sup>=0.906</p>

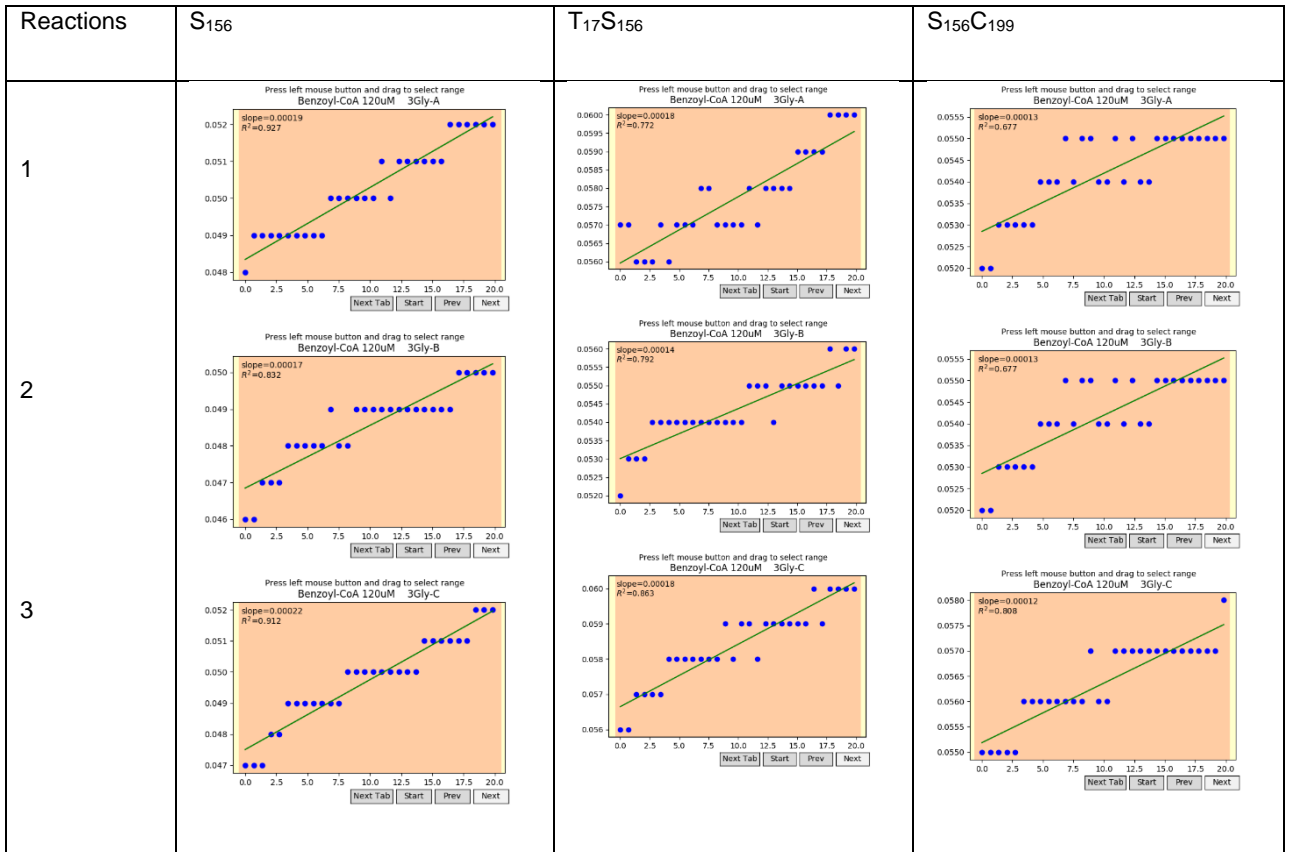
### Benzoyl-CoA : 120μM and Glycine 1mM



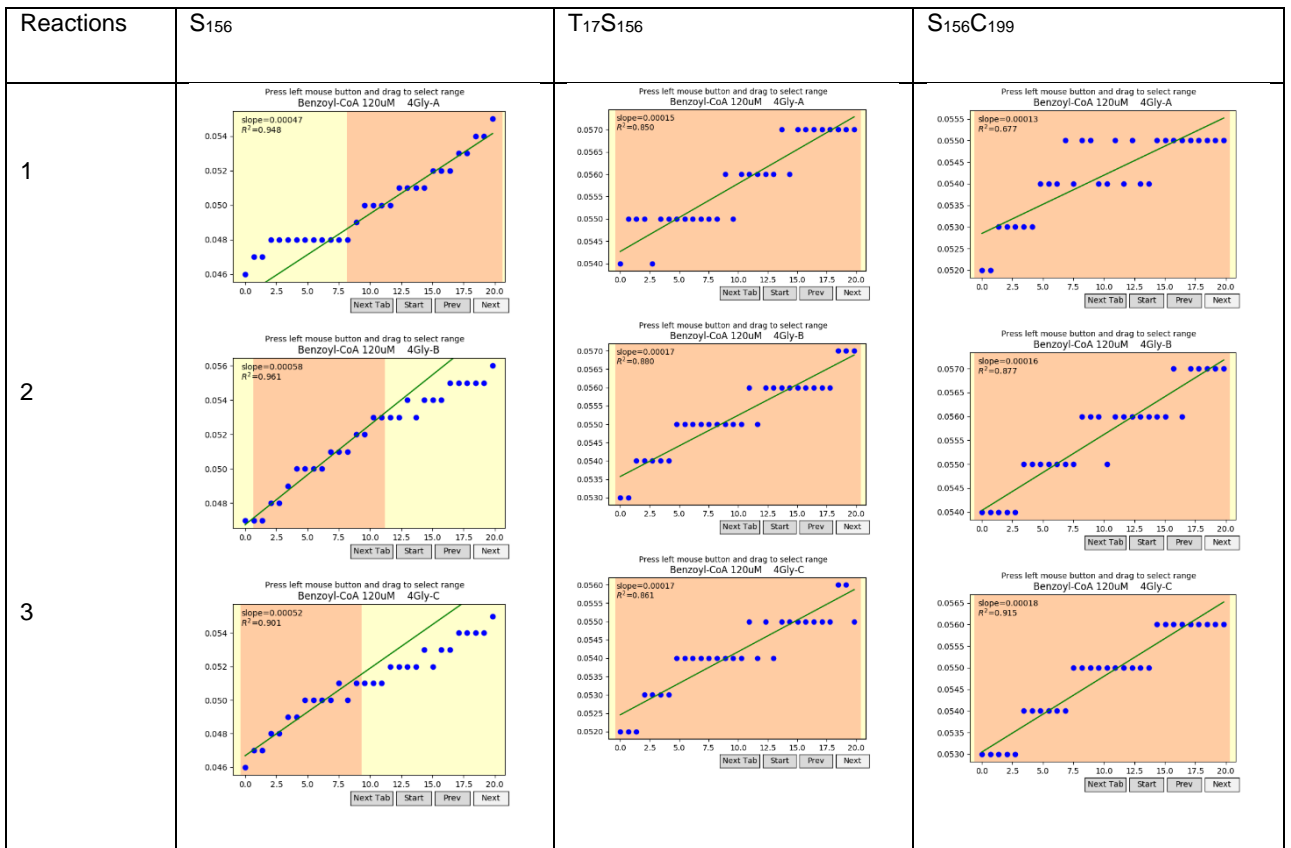
### Benzoyl-CoA : 120μM and Glycine 2mM



## Benzoyl-CoA : 120 $\mu$ M and Glycine 3mM



## Benzoyl-CoA : 120 $\mu$ M and Glycine 4mM



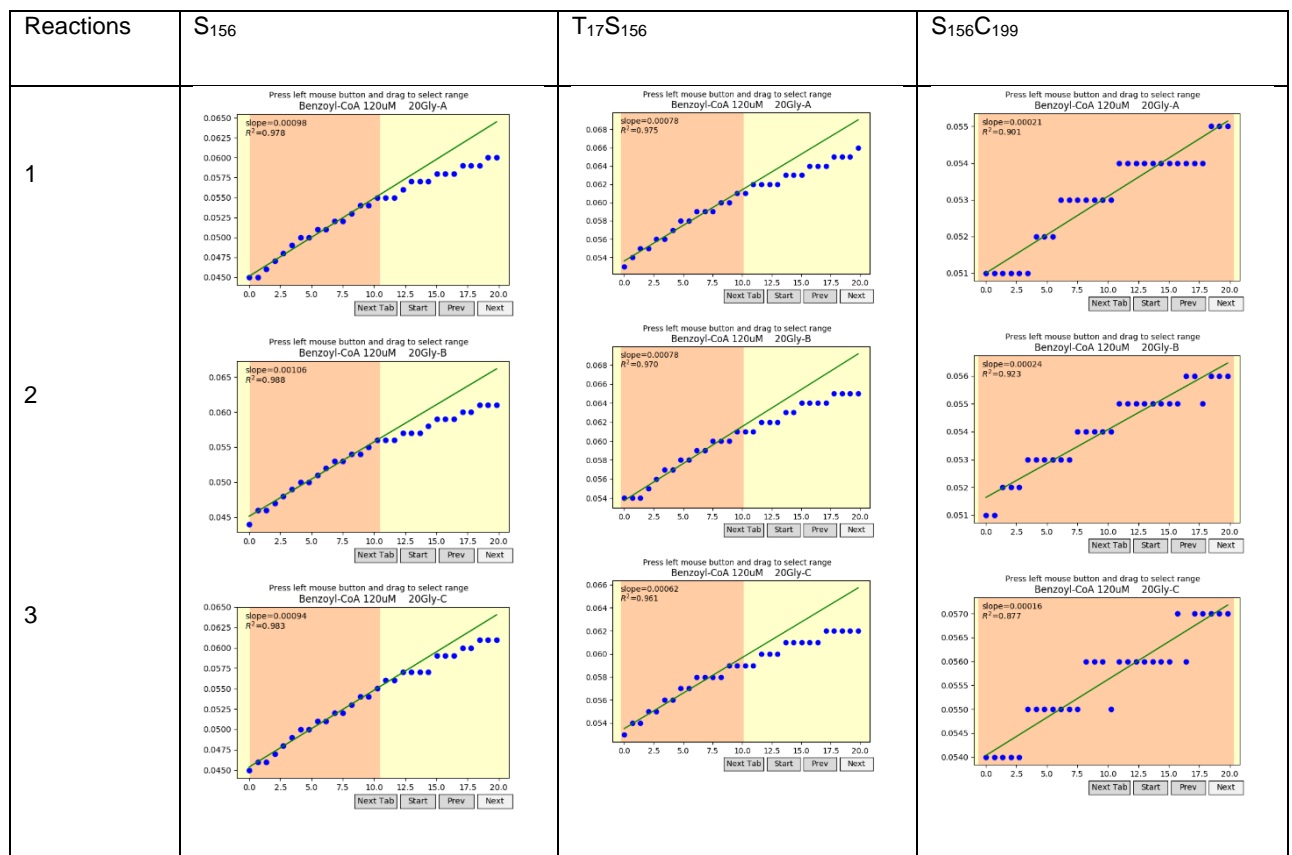
# Benzoyl-CoA : 120μM and Glycine 5mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 5Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 5Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 5Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 5Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 5Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 5Gly-C</p>

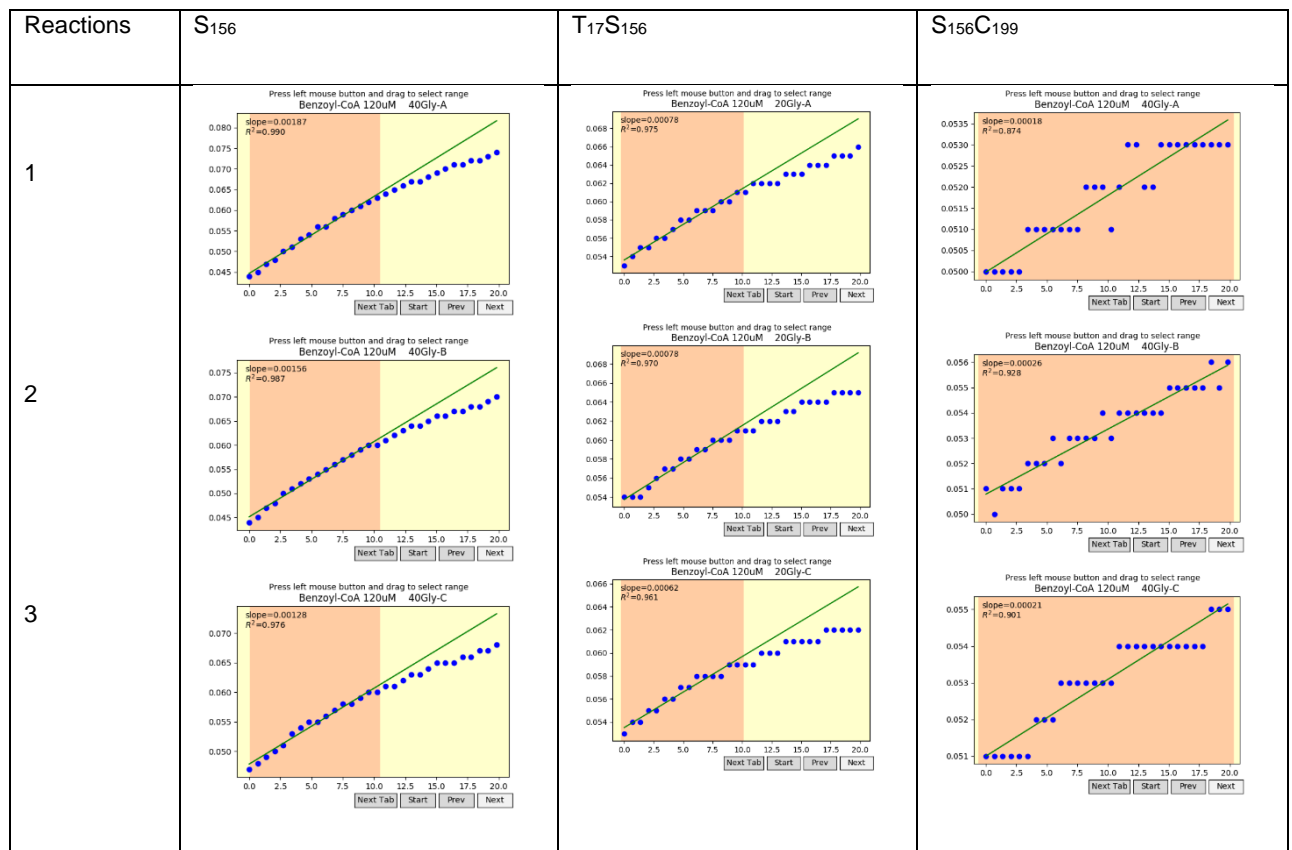
# Benzoyl-CoA : 120μM and Glycine 10mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120μM 10Gly-C</p>

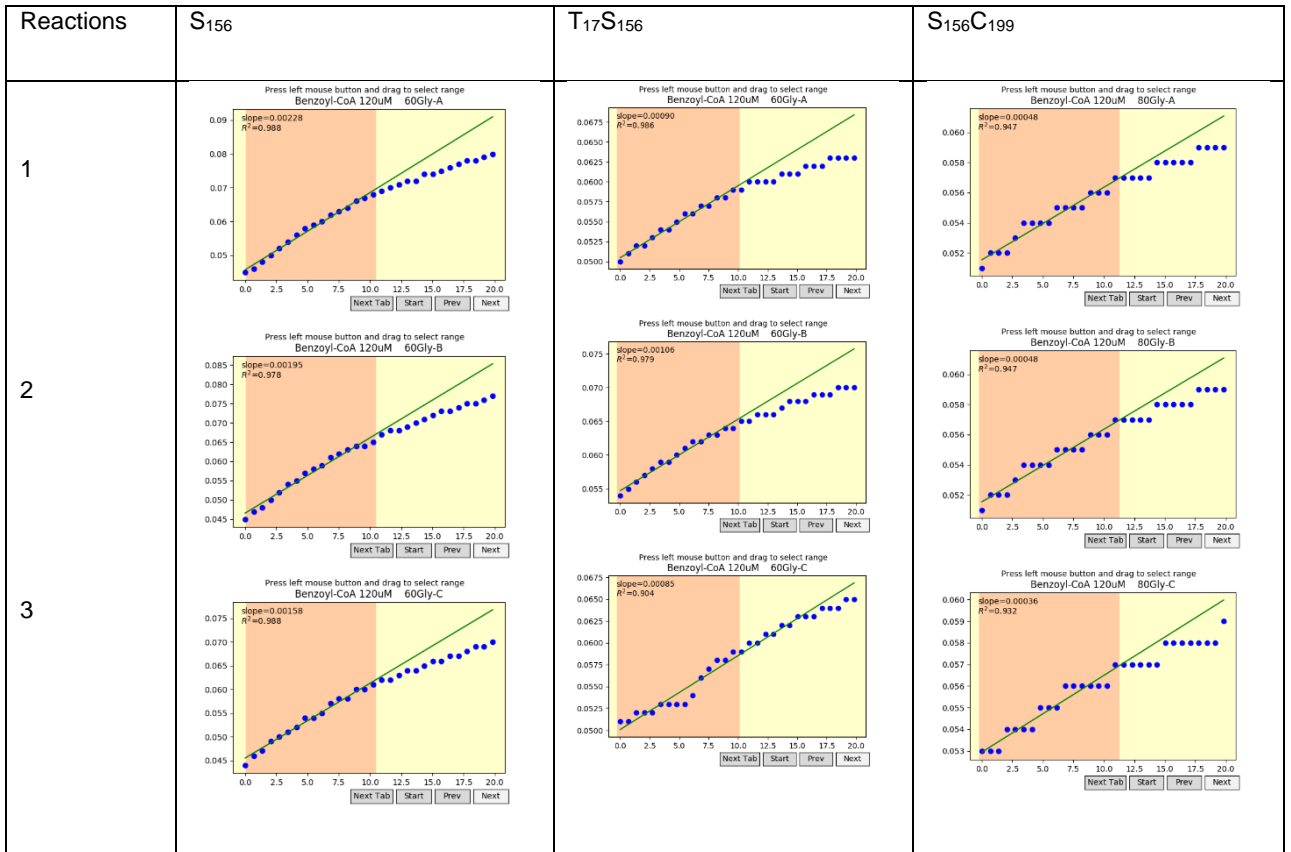
## Benzoyl-CoA : 120 $\mu$ M and Glycine 20mM



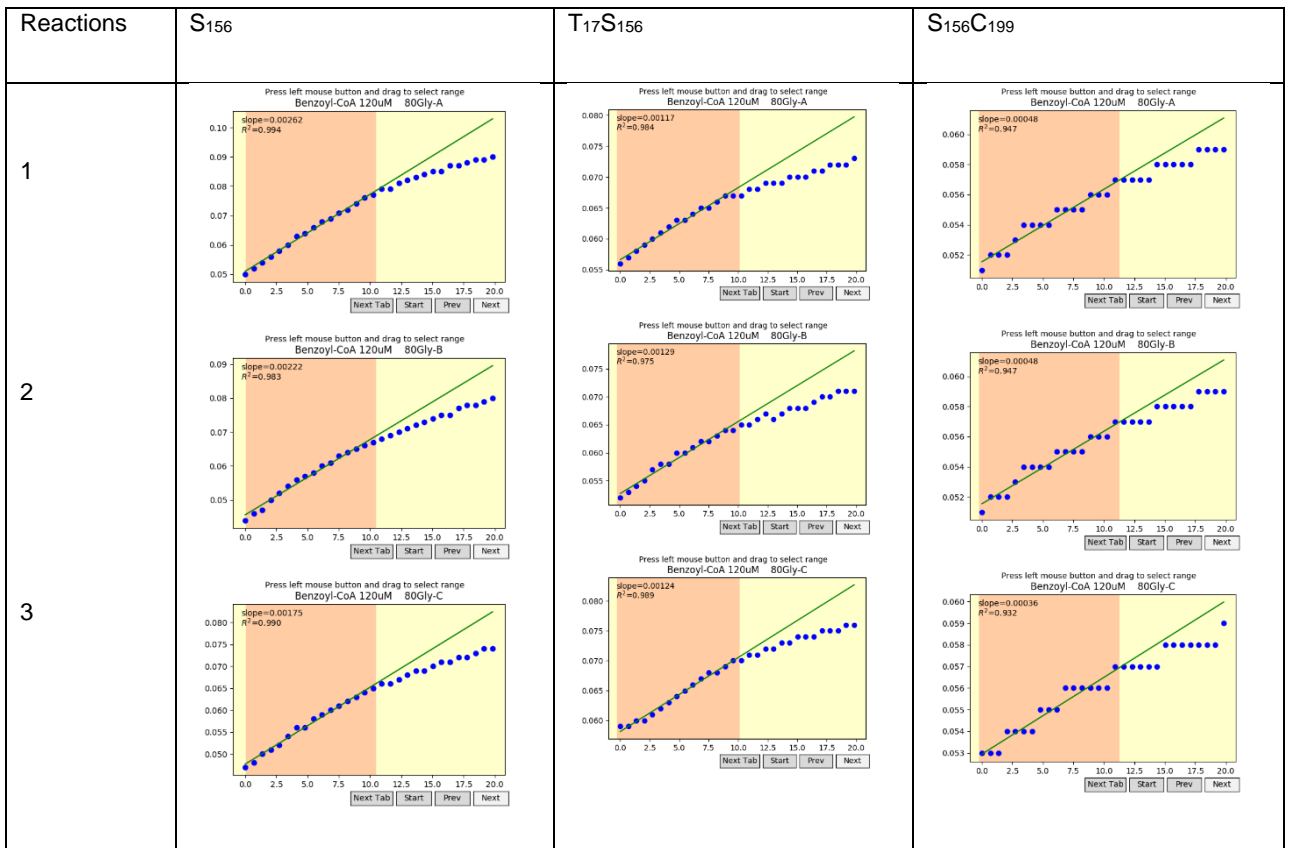
## Benzoyl-CoA : 120 $\mu$ M and Glycine 40mM



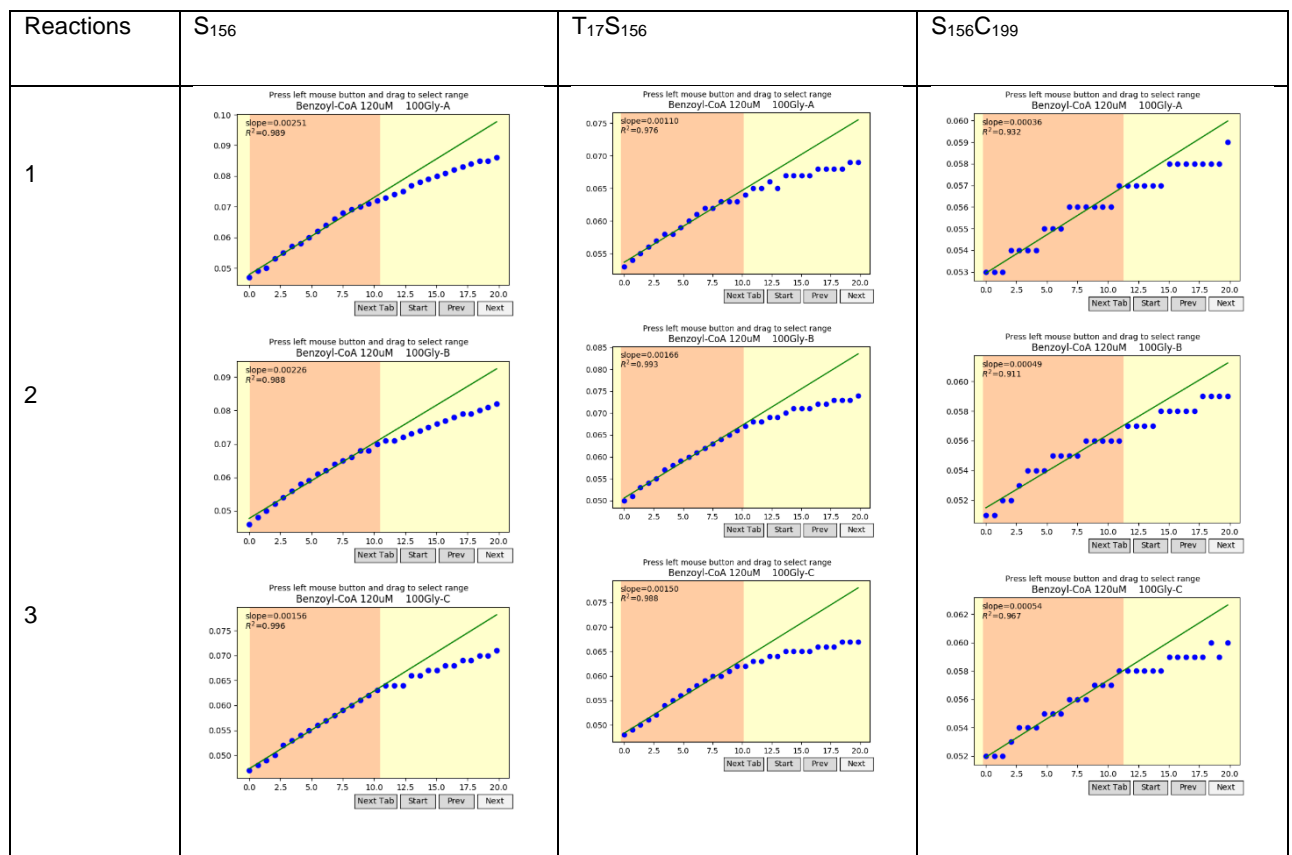
## Benzoyl-CoA : 120 $\mu$ M and Glycine 60mM



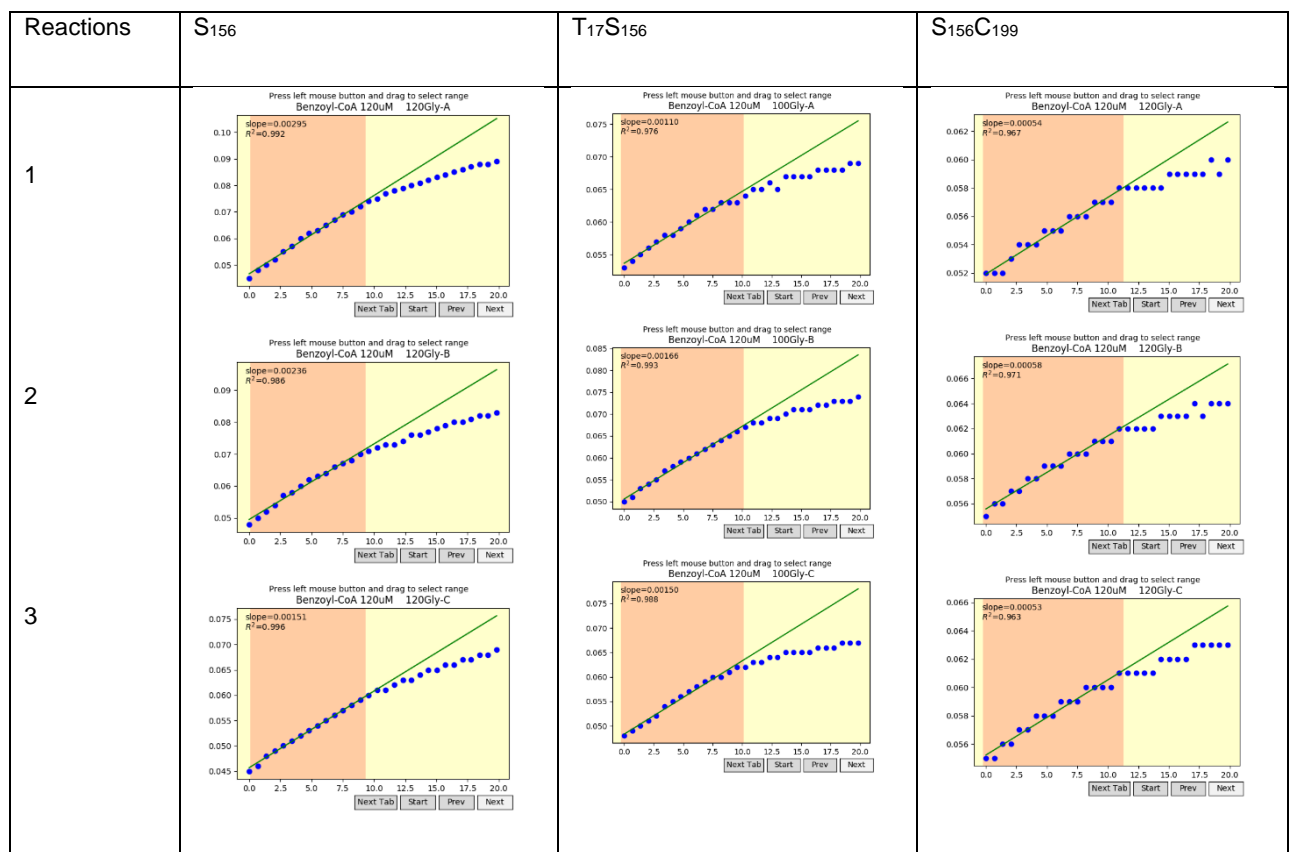
## Benzoyl-CoA : 120 $\mu$ M and Glycine 80mM



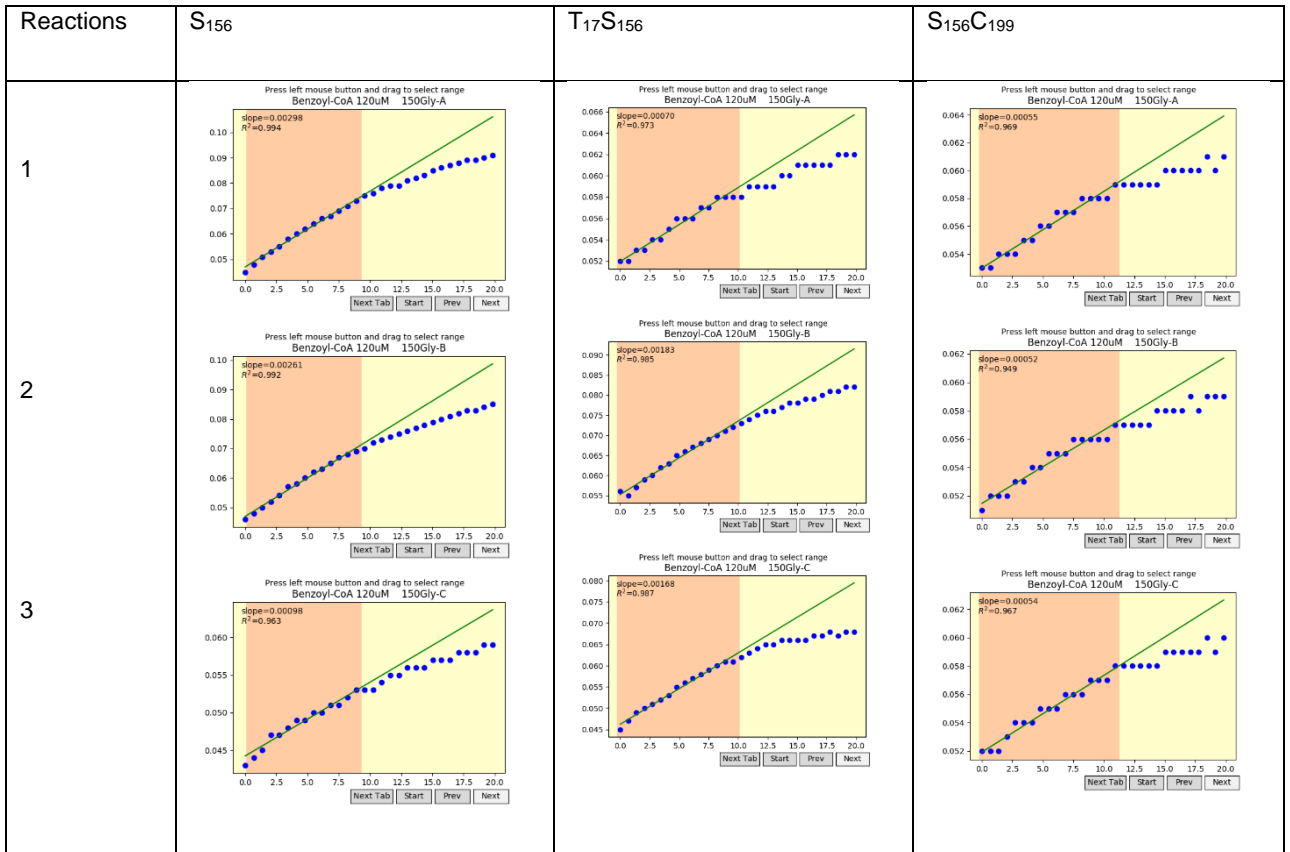
### Benzoyl-CoA : 120μM and Glycine 100mM



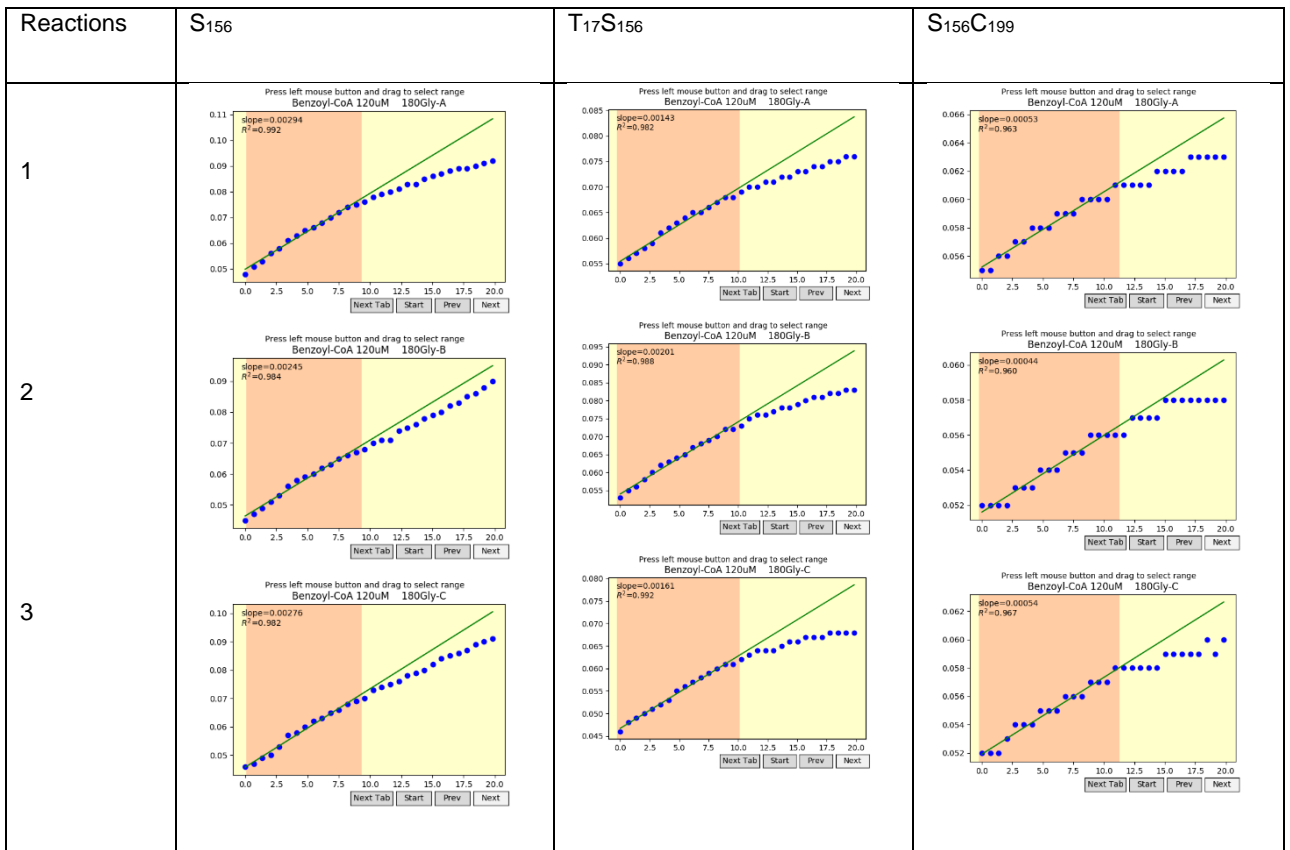
### Benzoyl-CoA : 120μM and Glycine 120mM



### Benzoyl-CoA : 120μM and Glycine 150mM



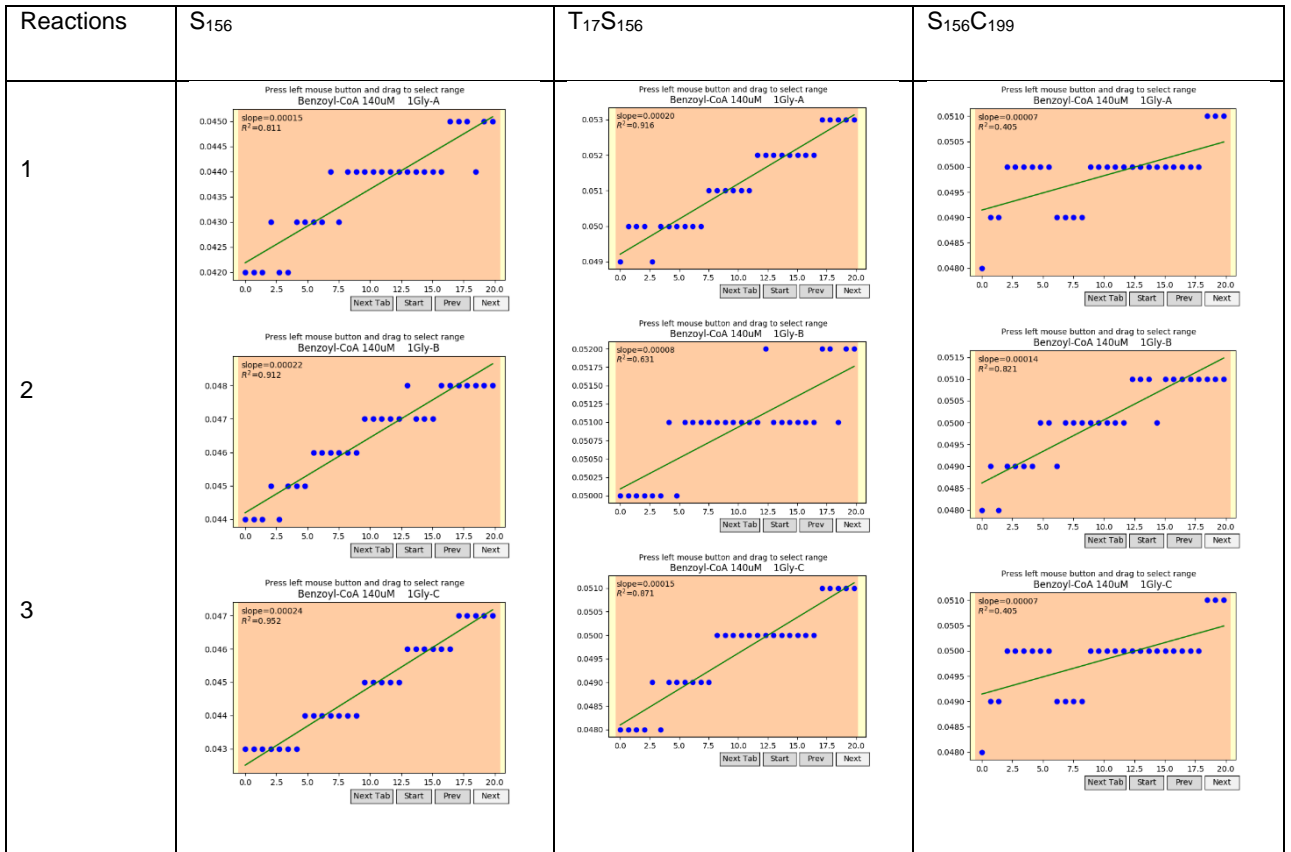
### Benzoyl-CoA : 120μM and Glycine 180mM



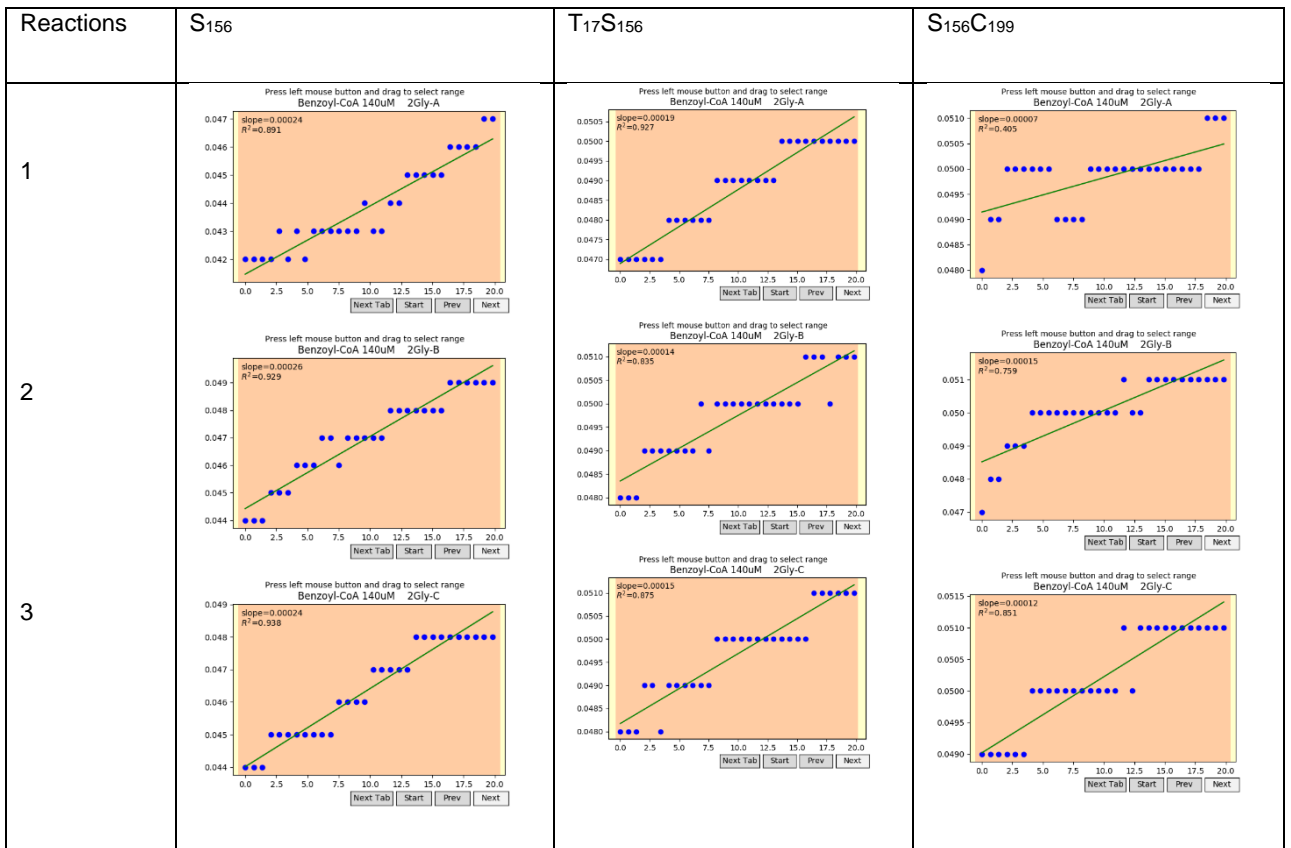
**Benzoyl-CoA : 120 $\mu$ M and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-A</p> <p>slope=0.00309 R<sup>2</sup>=0.988</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-A</p> <p>slope=0.00119 R<sup>2</sup>=0.981</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-A</p> <p>slope=0.00056 R<sup>2</sup>=0.909</p> <p>Next Tab Start Prev Next</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-B</p> <p>slope=0.00255 R<sup>2</sup>=0.985</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-B</p> <p>slope=0.00188 R<sup>2</sup>=0.981</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-B</p> <p>slope=0.00046 R<sup>2</sup>=0.944</p> <p>Next Tab Start Prev Next</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-C</p> <p>slope=0.00269 R<sup>2</sup>=0.981</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-C</p> <p>slope=0.00137 R<sup>2</sup>=0.978</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 120<math>\mu</math>M 200Gly-C</p> <p>slope=0.00056 R<sup>2</sup>=0.909</p> <p>Next Tab Start Prev Next</p>

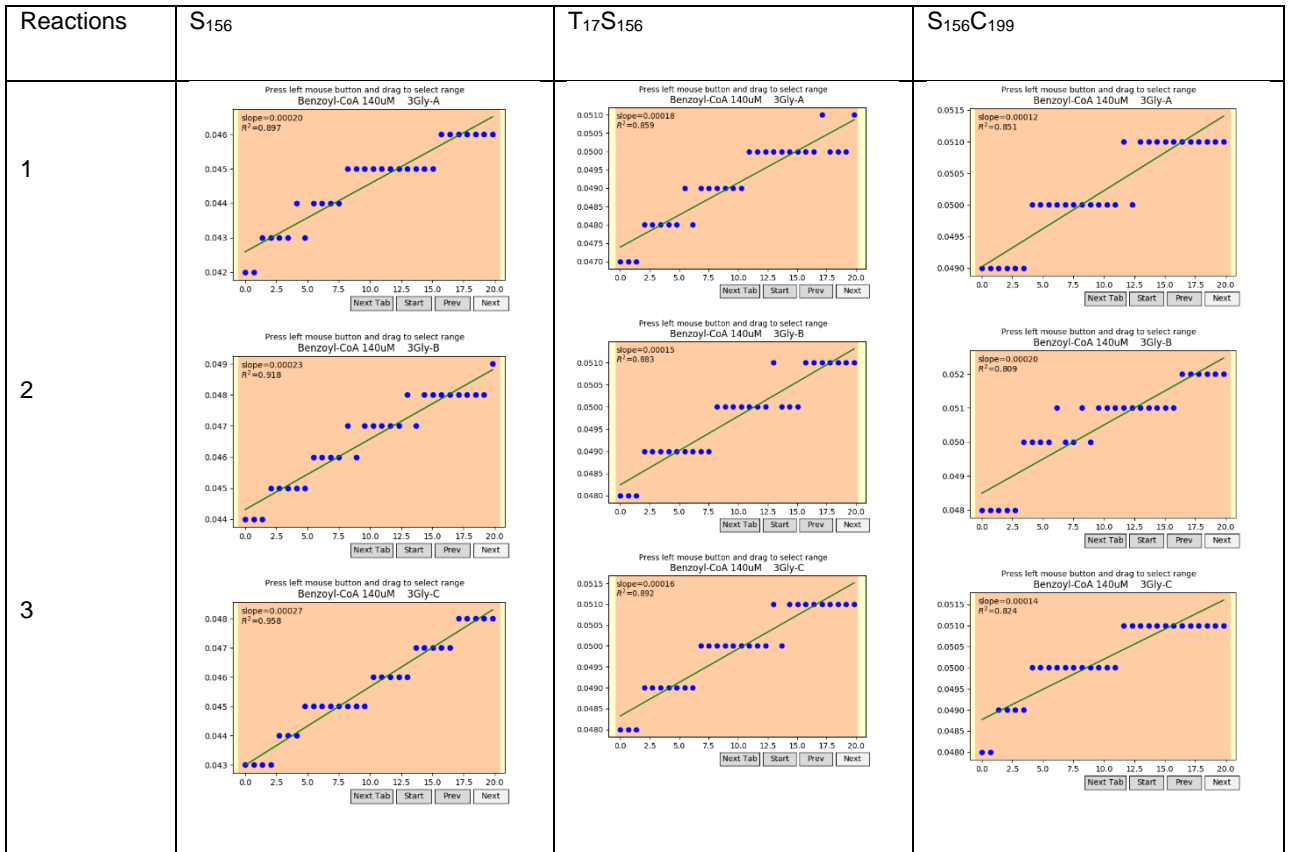
## Benzoyl-CoA : 140 $\mu$ M and Glycine 1mM



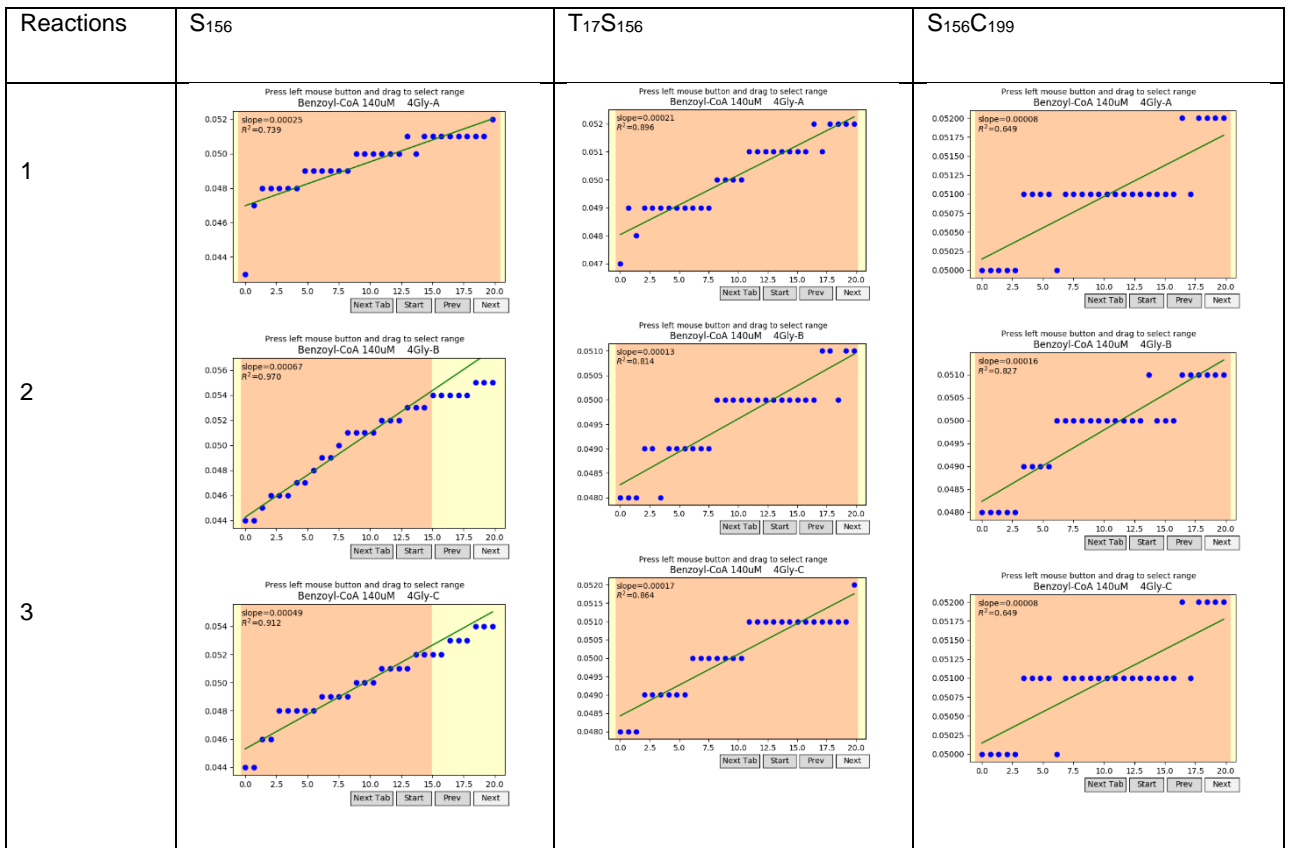
## Benzoyl-CoA : 140 $\mu$ M and Glycine 2mM



## Benzoyl-CoA : 140μM and Glycine 3mM



## Benzoyl-CoA : 140μM and Glycine 4mM



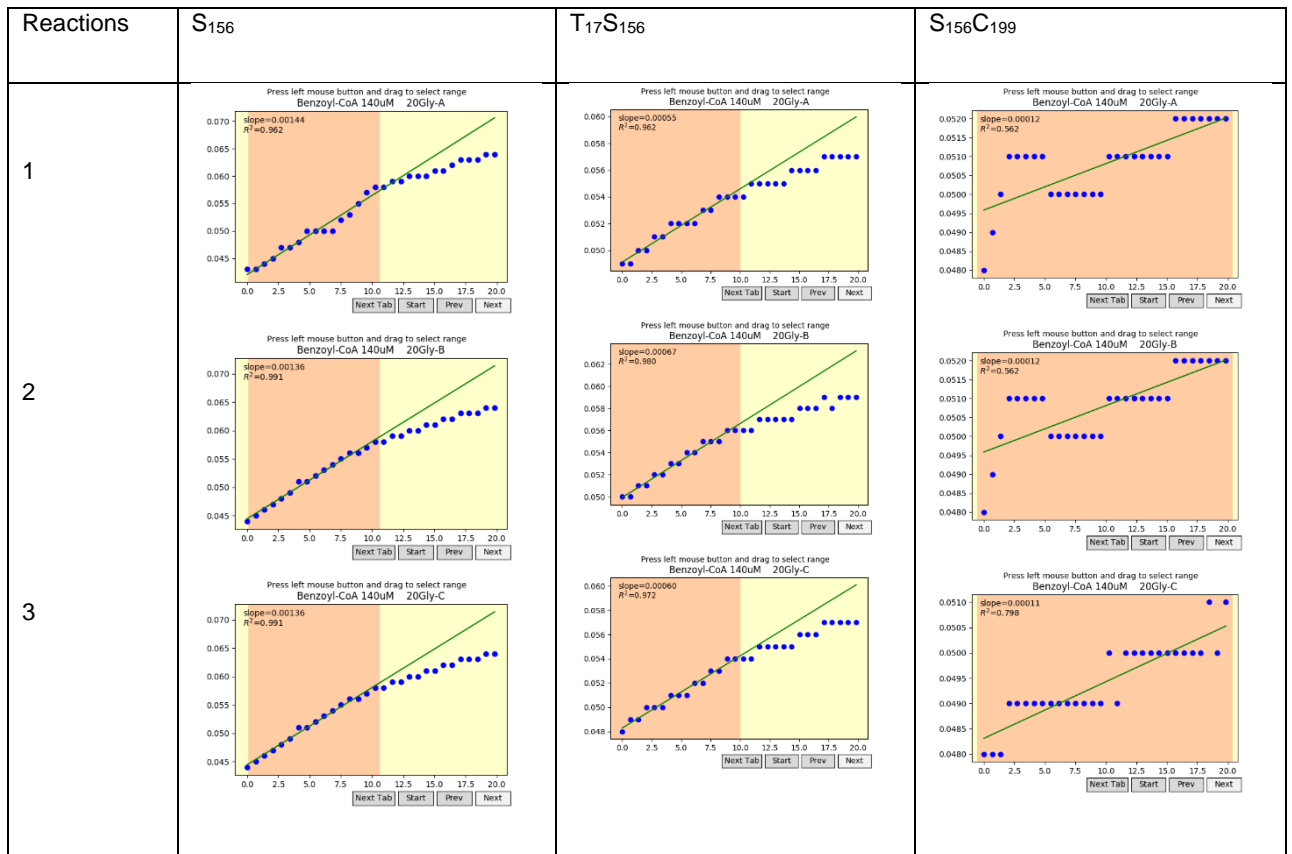
## Benzoyl-CoA : 140μM and Glycine 5mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 5Gly-C</p>

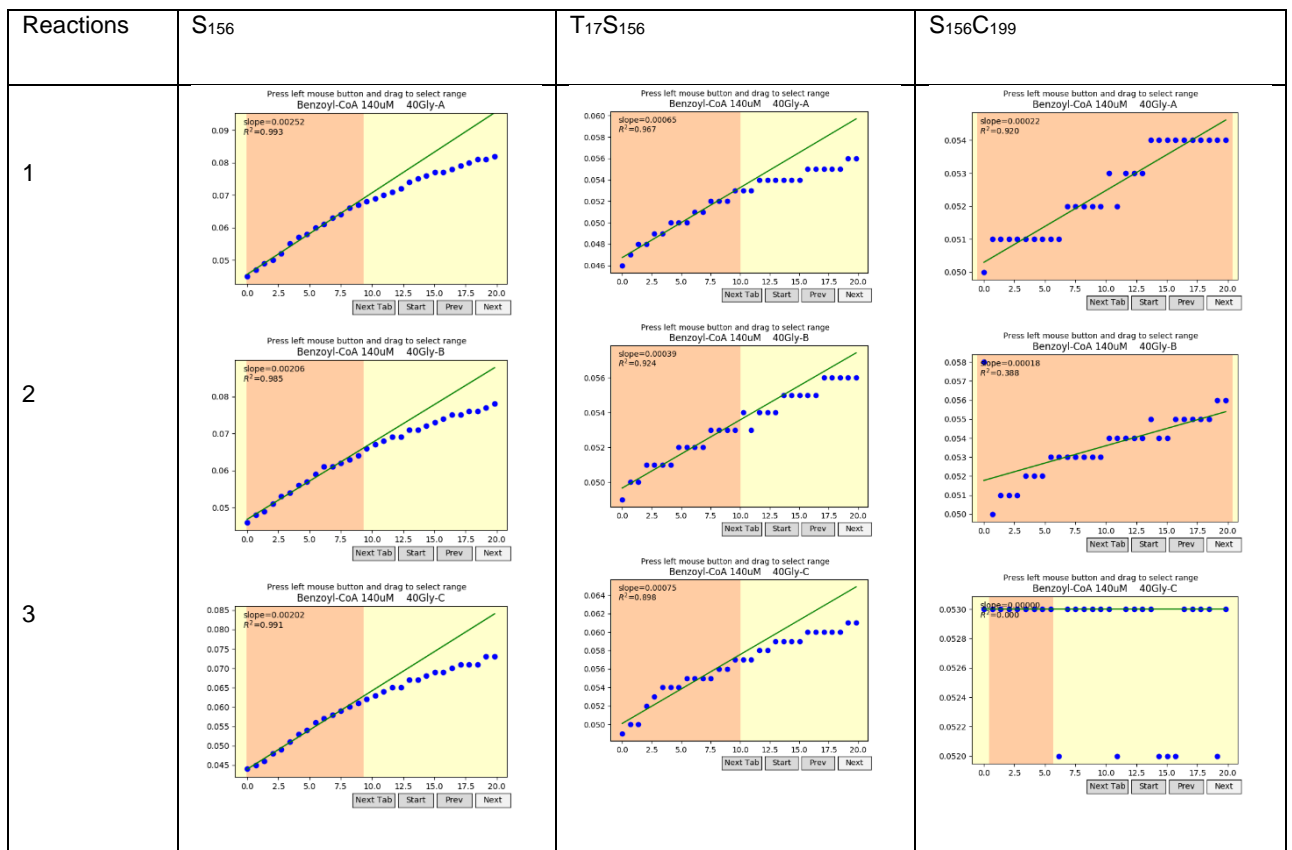
## Benzoyl-CoA : 140μM and Glycine 10mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140μM 10Gly-C</p>

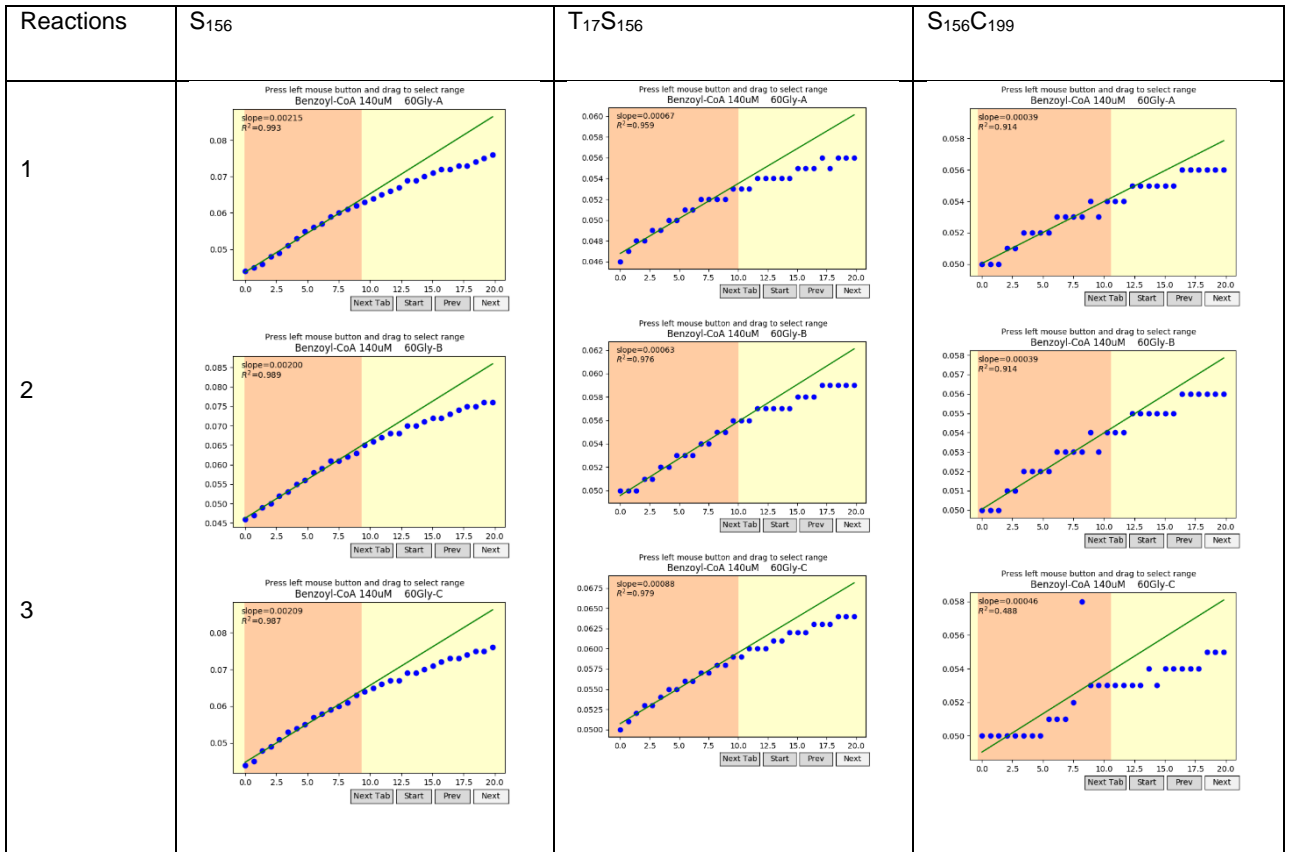
## Benzoyl-CoA : 140μM and Glycine 20mM



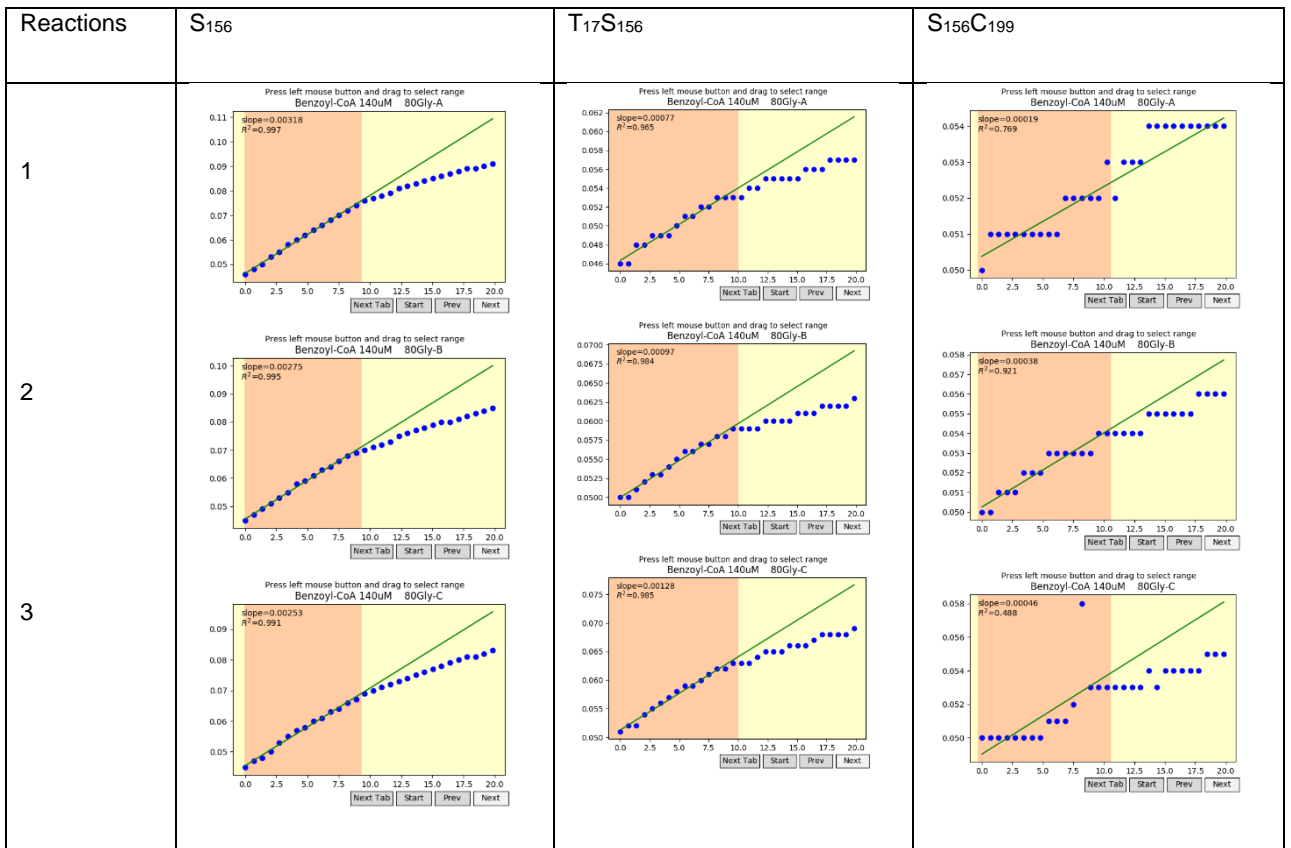
## Benzoyl-CoA : 140μM and Glycine 40mM



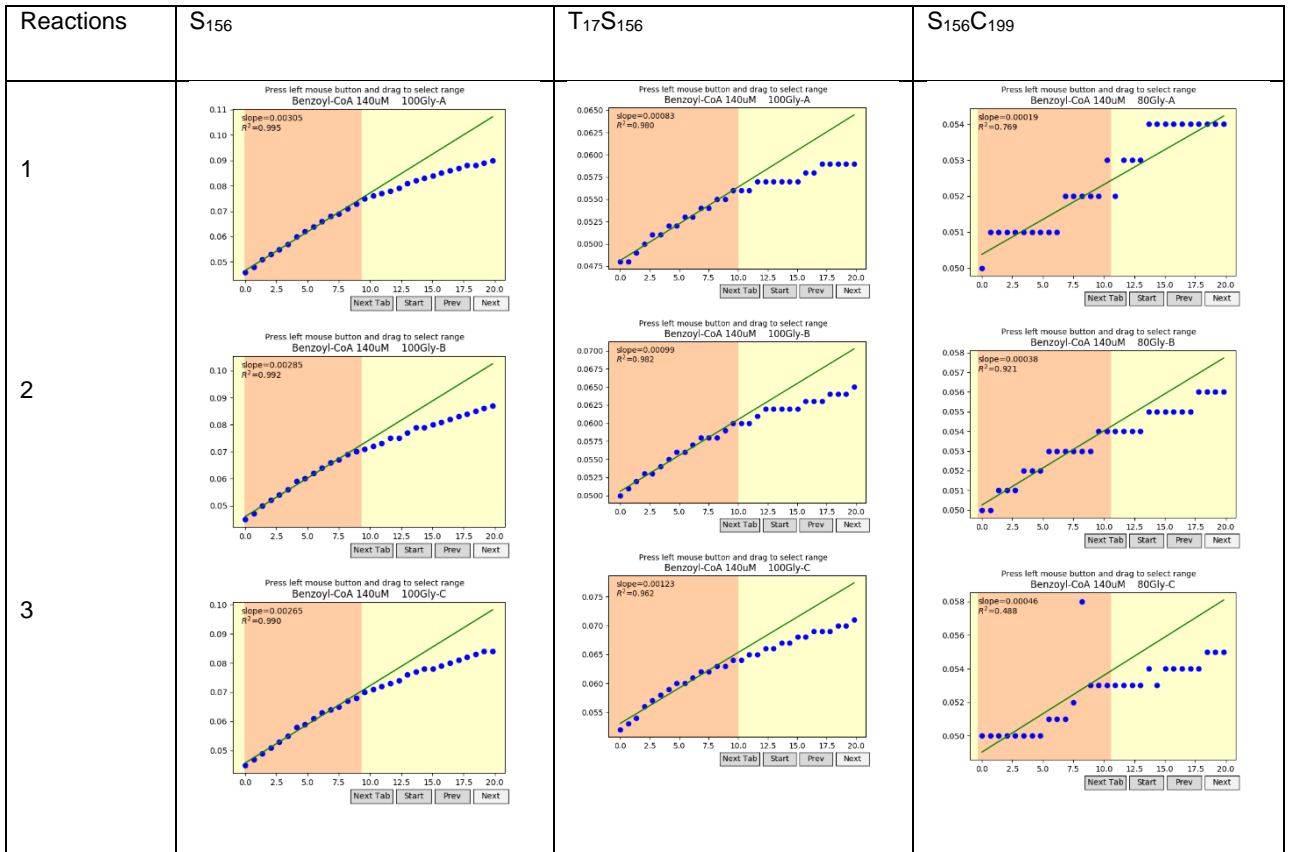
## Benzoyl-CoA : 140μM and Glycine 60mM



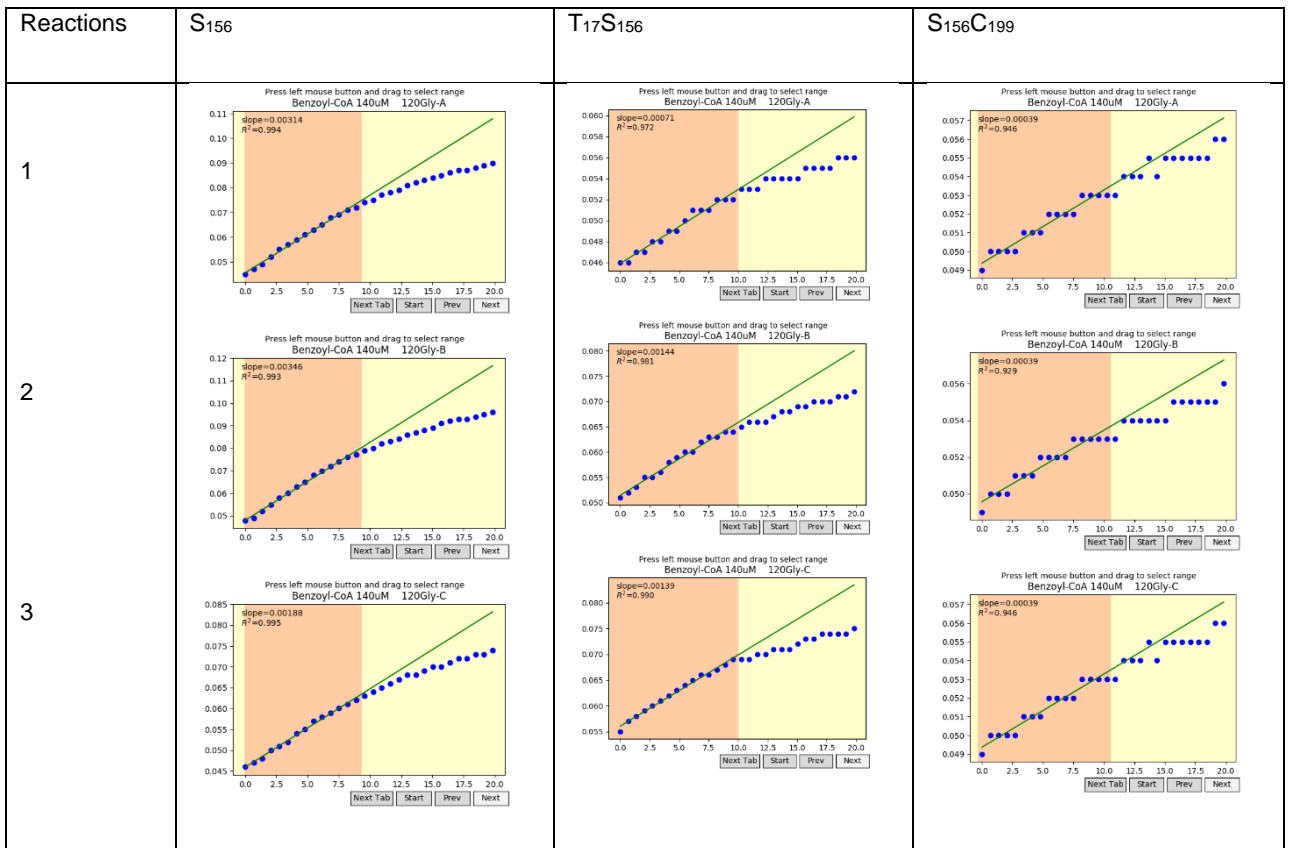
## Benzoyl-CoA : 140μM and Glycine 80mM



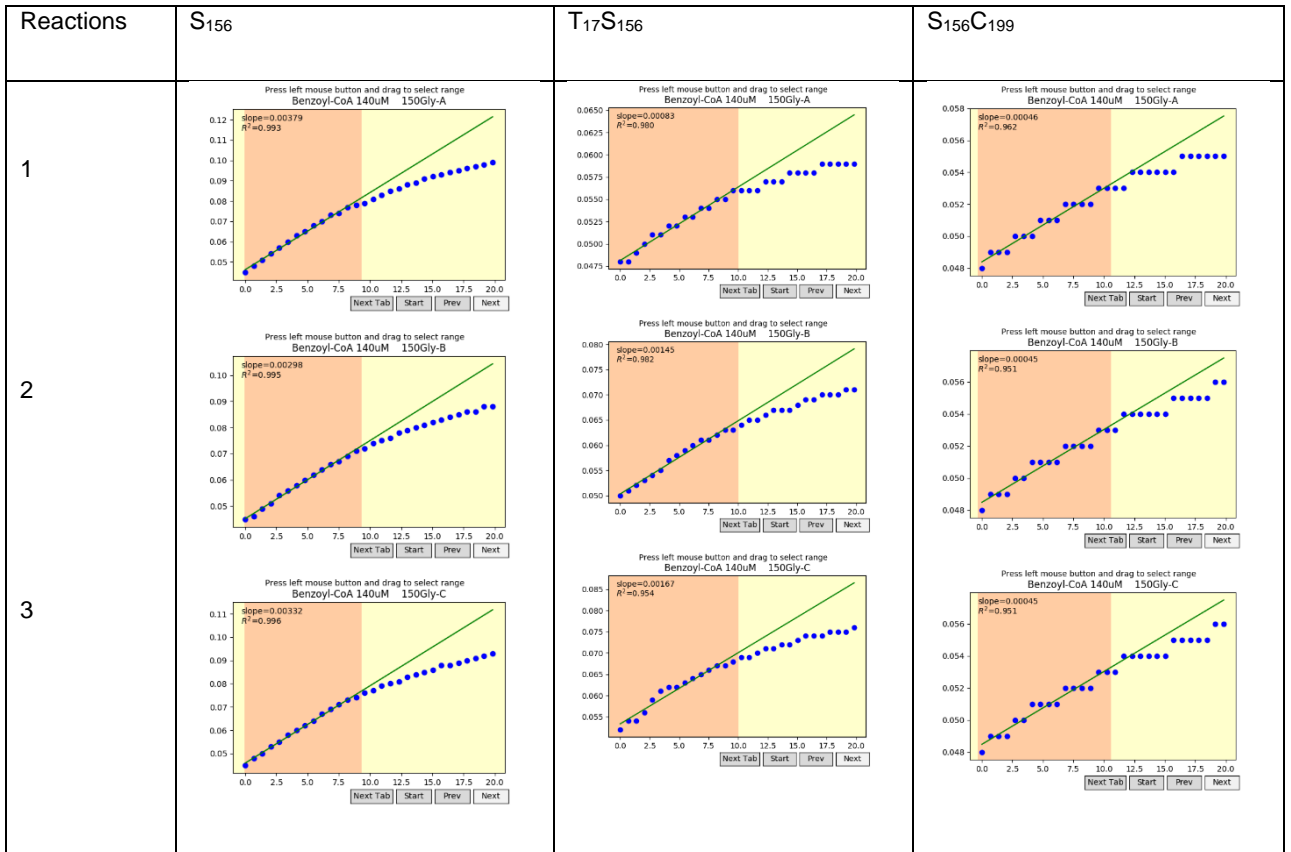
**Benzoyl-CoA : 140μM and Glycine 100mM**



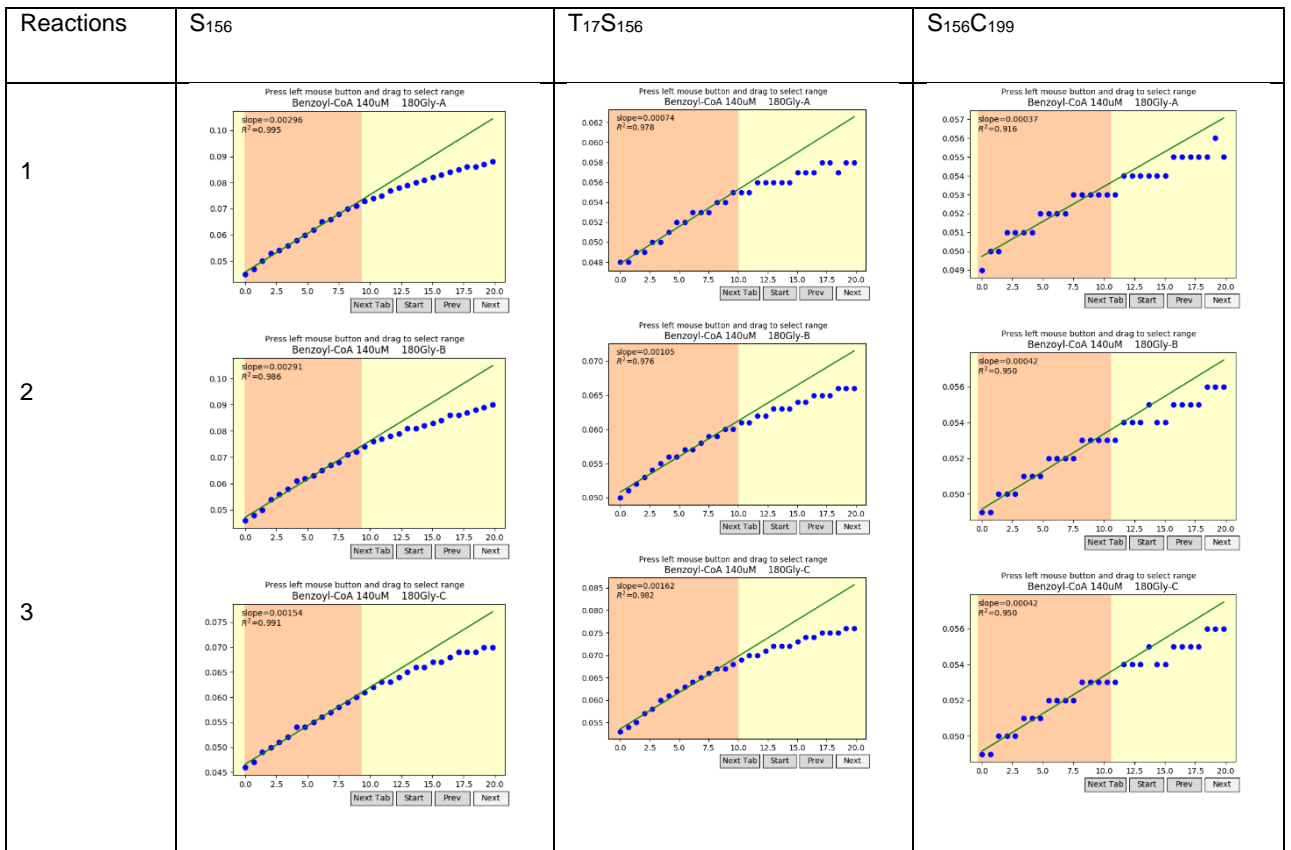
**Benzoyl-CoA : 140μM and Glycine 120mM**



**Benzoyl-CoA : 140μM and Glycine 150mM**



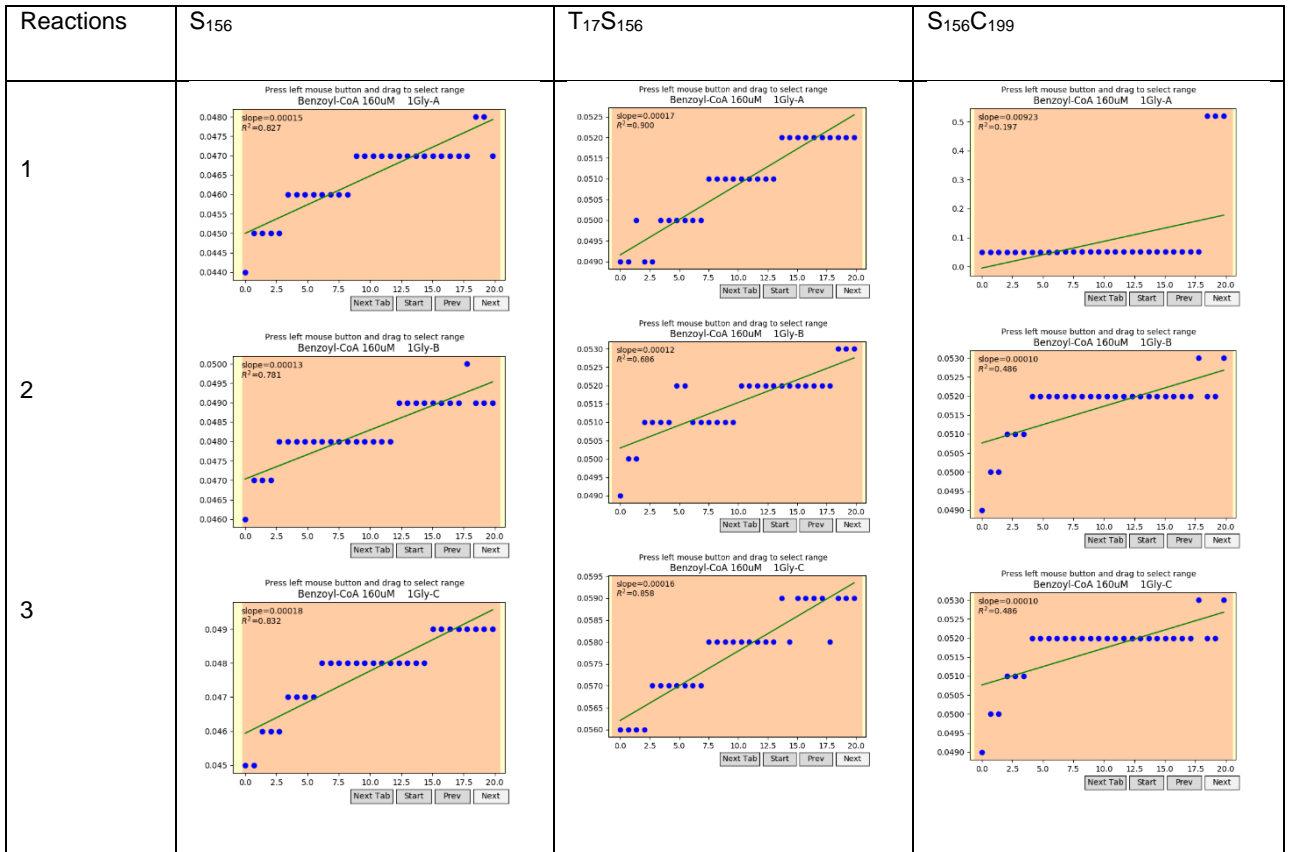
**Benzoyl-CoA : 140μM and Glycine 180mM**



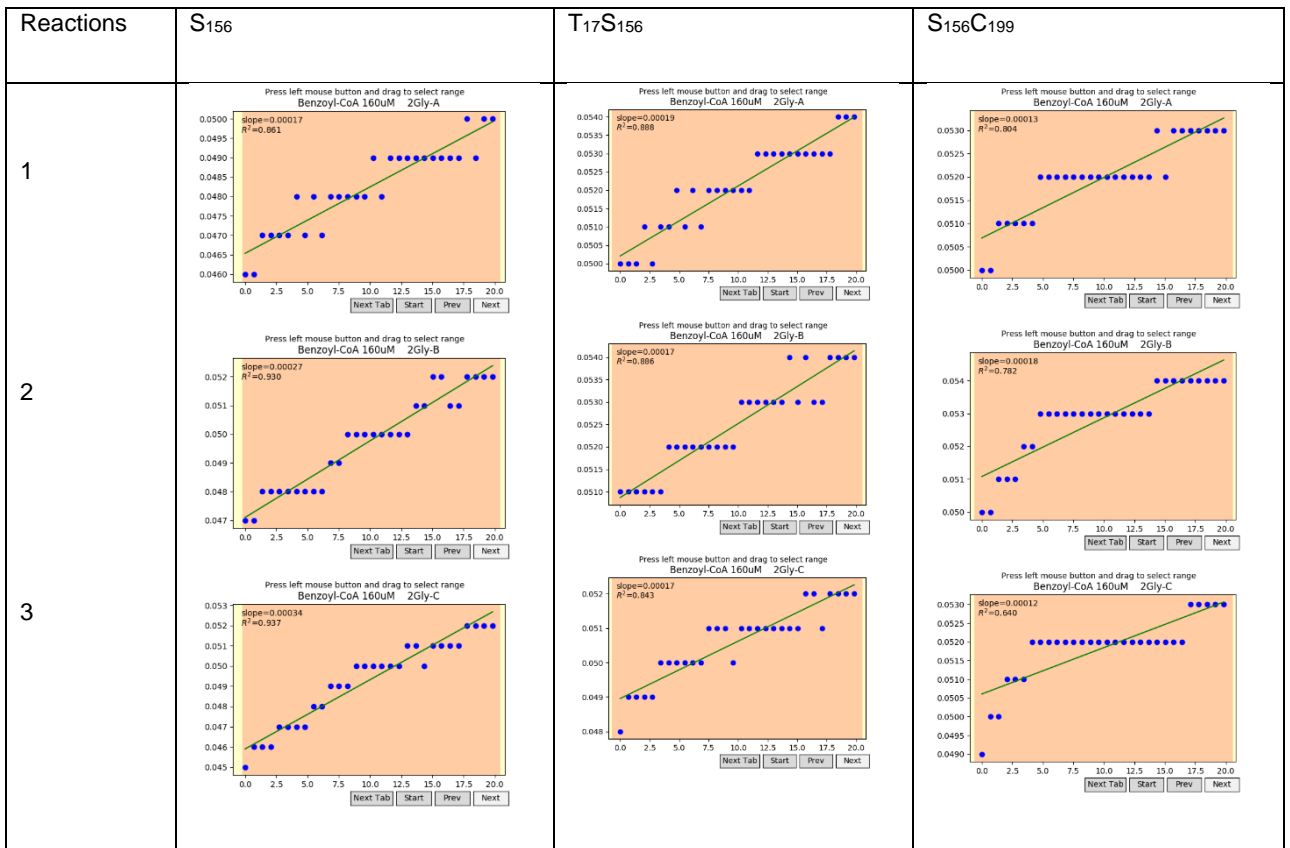
**Benzoyl-CoA : 140 $\mu$ M and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-A</p> <p>slope=0.00368 R<sup>2</sup>=0.992</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-A</p> <p>slope=0.00050 R<sup>2</sup>=0.957</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-A</p> <p>slope=0.0018 R<sup>2</sup>=0.081</p> <p>Next Tab Start Prev Next</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-B</p> <p>slope=0.00322 R<sup>2</sup>=0.991</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-B</p> <p>slope=0.00133 R<sup>2</sup>=0.991</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-B</p> <p>slope=0.00039 R<sup>2</sup>=0.929</p> <p>Next Tab Start Prev Next</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-C</p> <p>slope=0.00332 R<sup>2</sup>=0.996</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-C</p> <p>slope=0.00146 R<sup>2</sup>=0.984</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 140<math>\mu</math>M 200Gly-C</p> <p>slope=0.0018 R<sup>2</sup>=0.081</p> <p>Next Tab Start Prev Next</p>

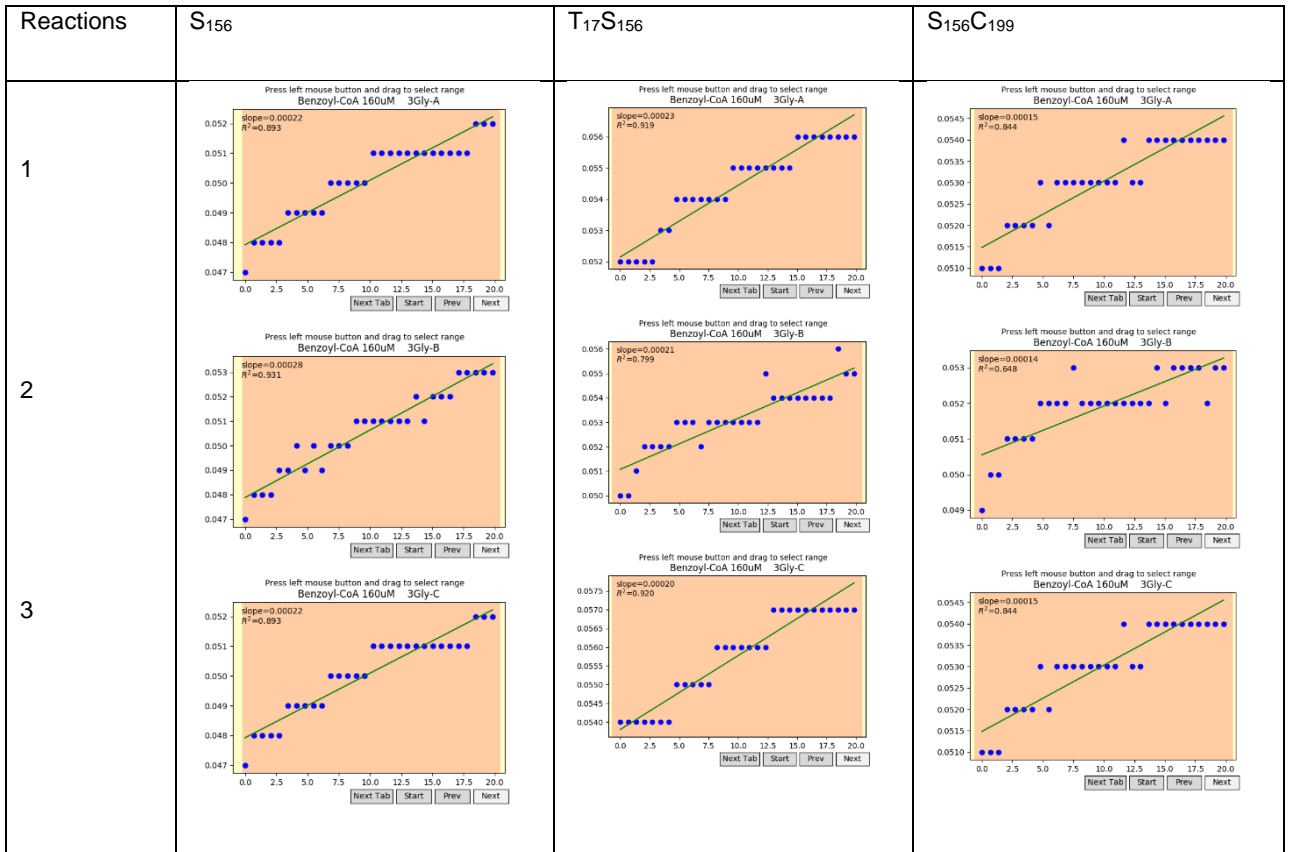
## Benzoyl-CoA : 160µM and Glycine 1mM



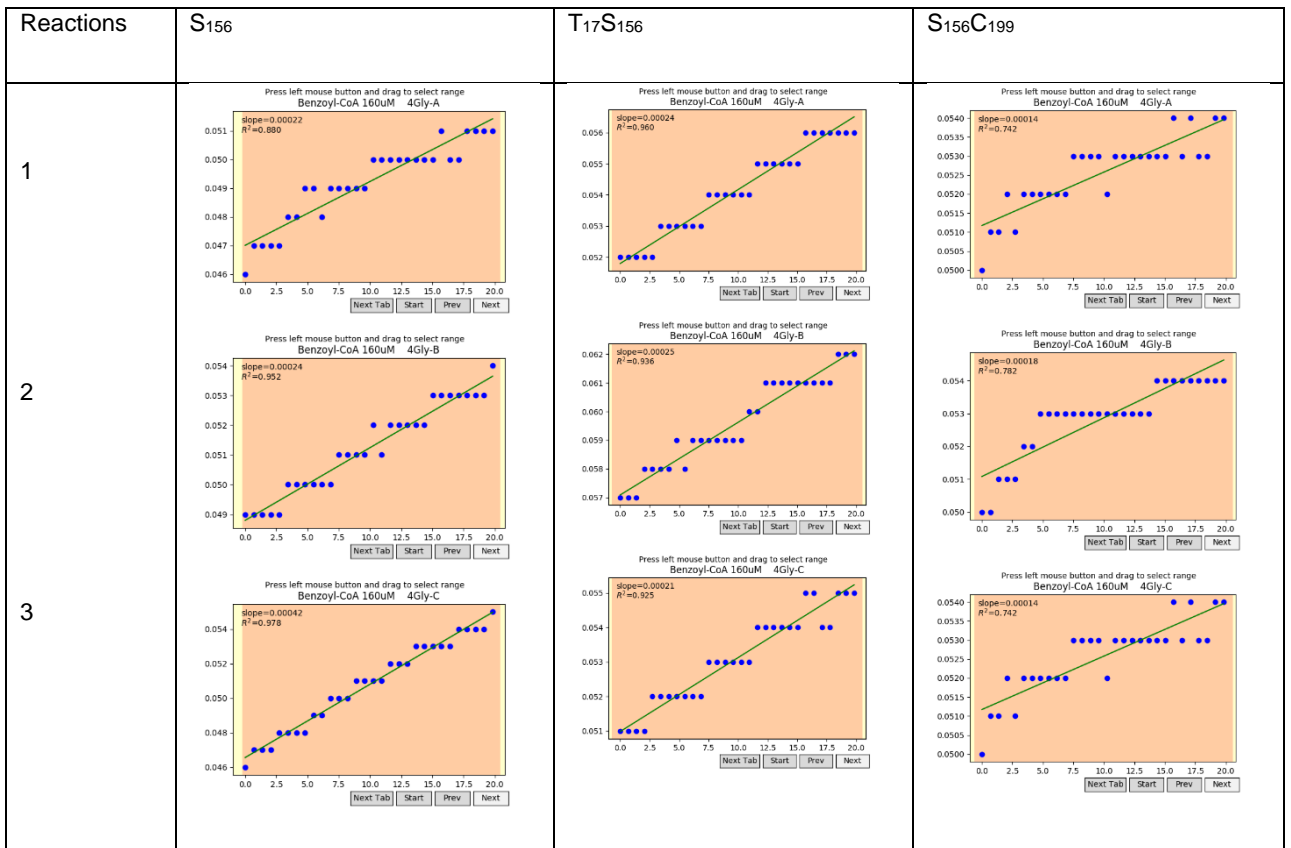
## Benzoyl-CoA : 160µM and Glycine 2mM



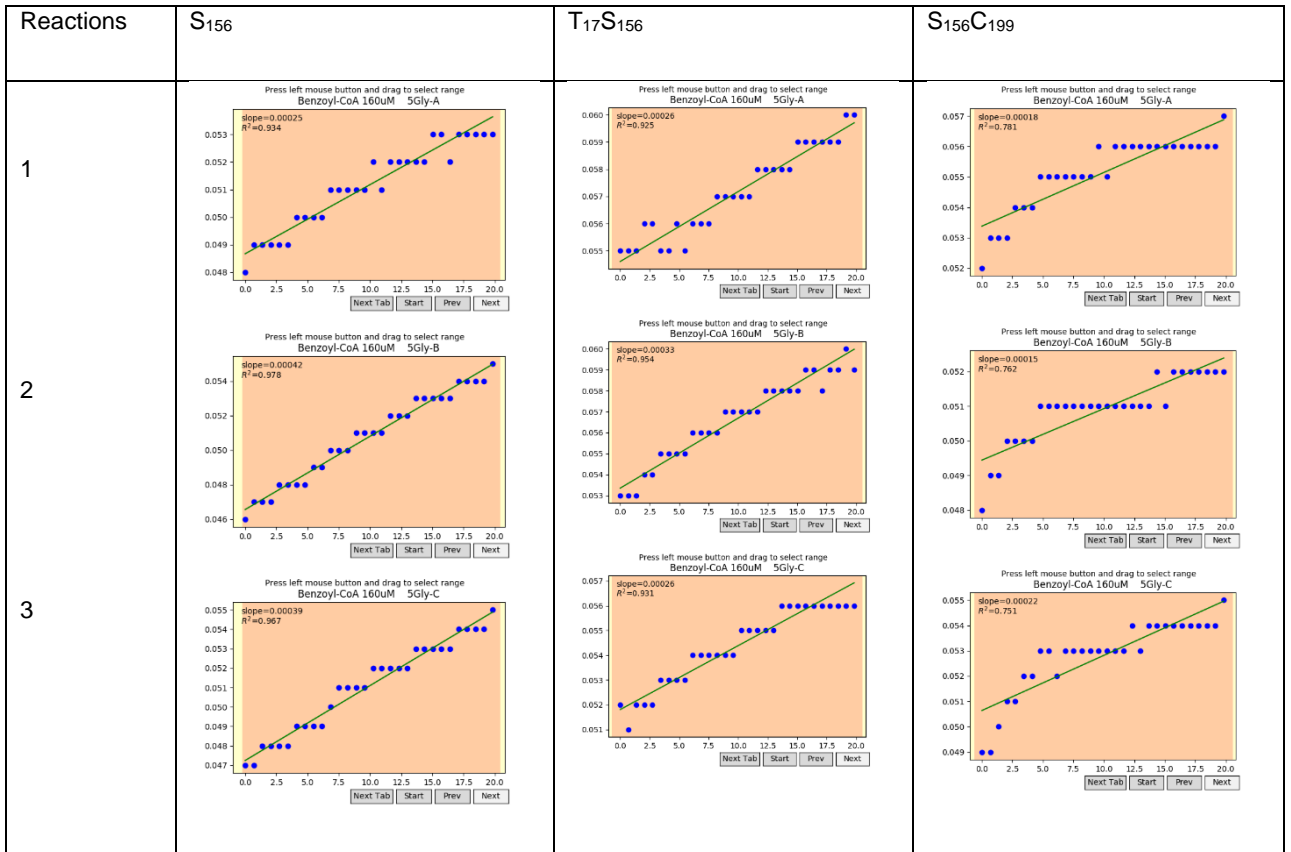
## Benzoyl-CoA : 160µM and Glycine 3mM



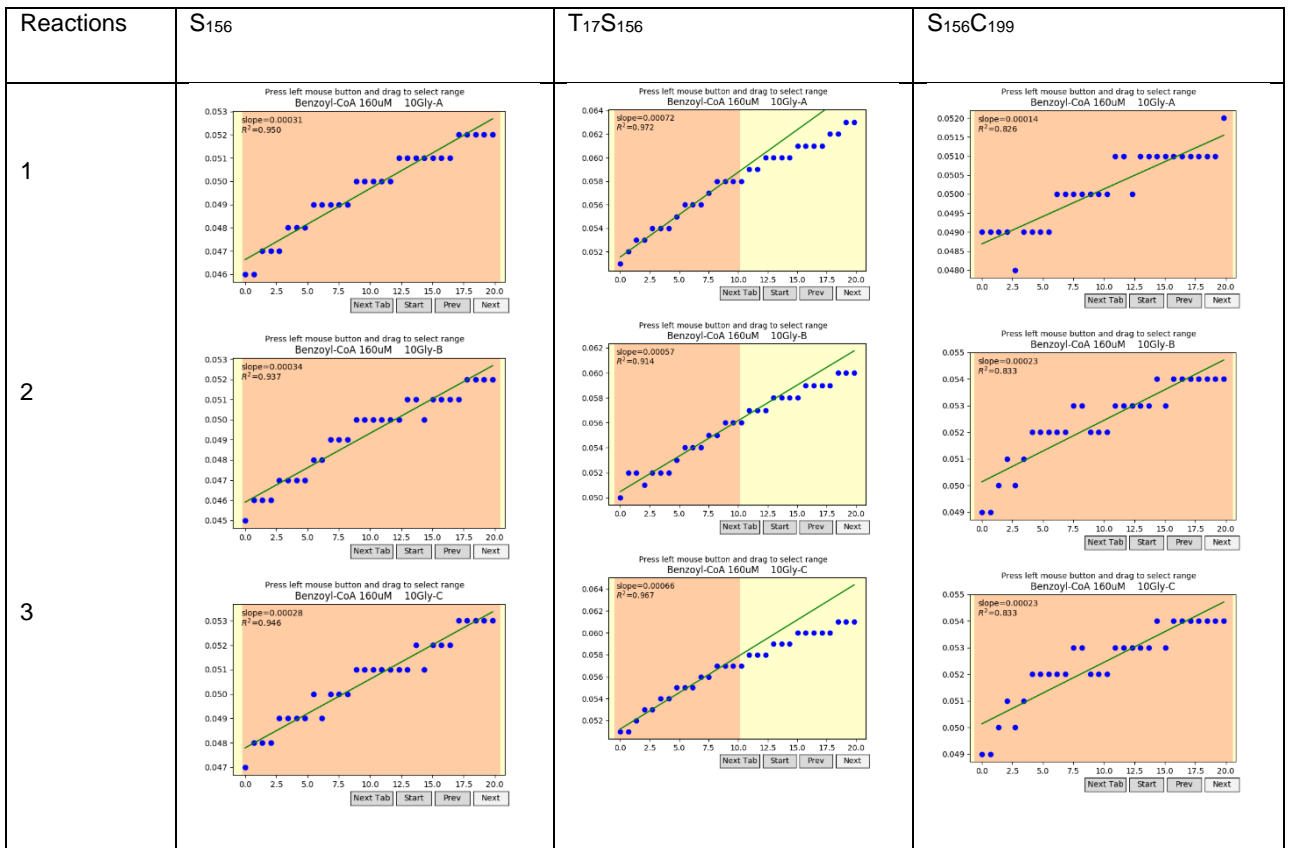
## Benzoyl-CoA : 160µM and Glycine 4mM



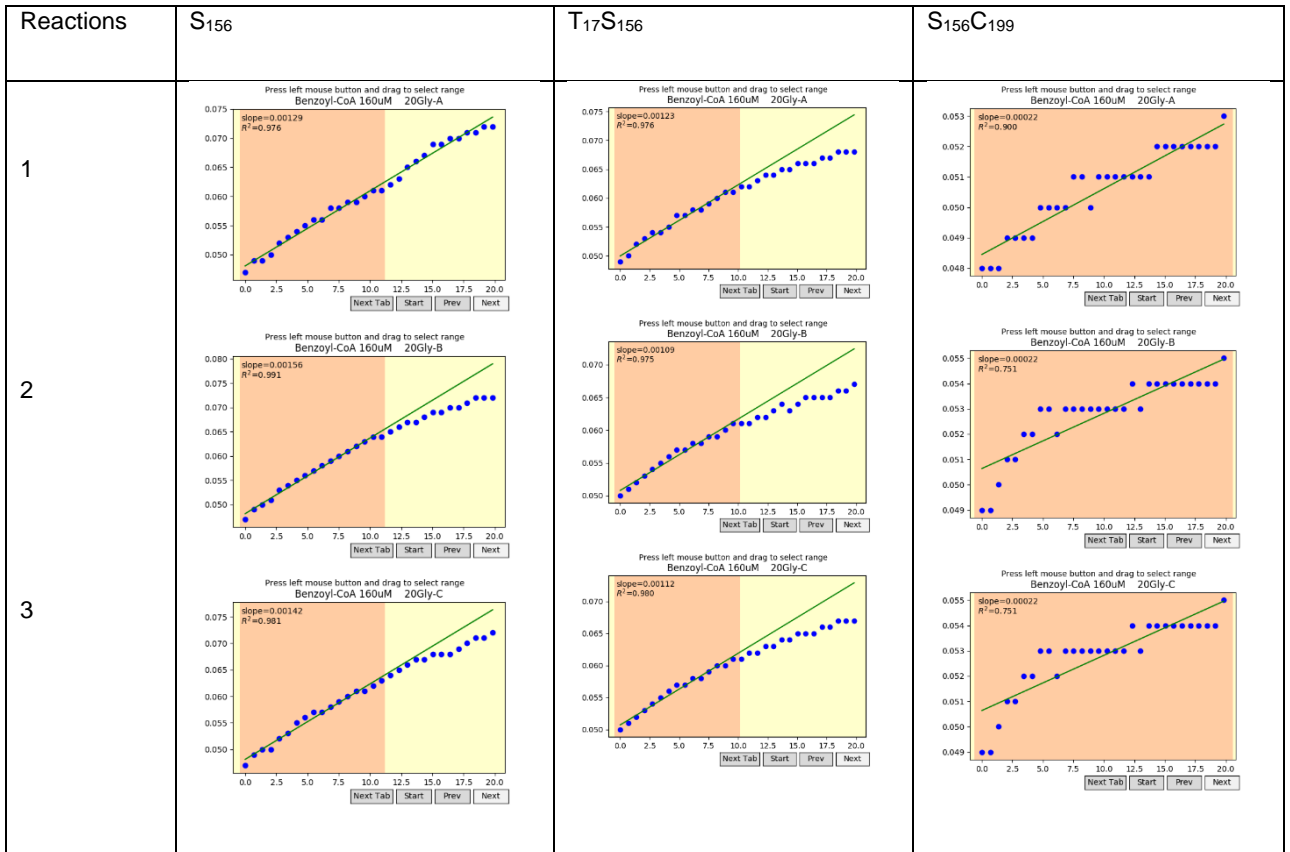
## Benzoyl-CoA : 160μM and Glycine 5mM



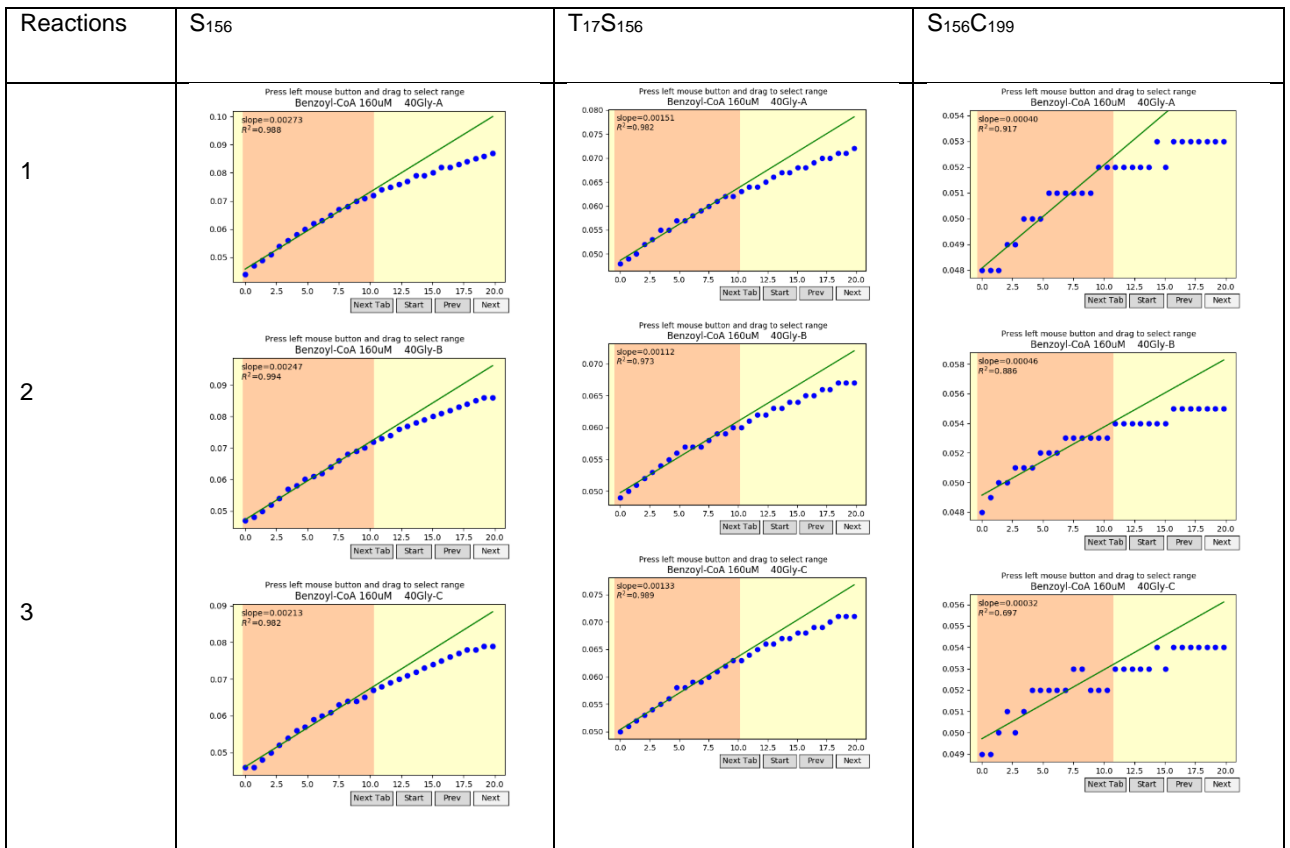
## Benzoyl-CoA : 160μM and Glycine 10mM



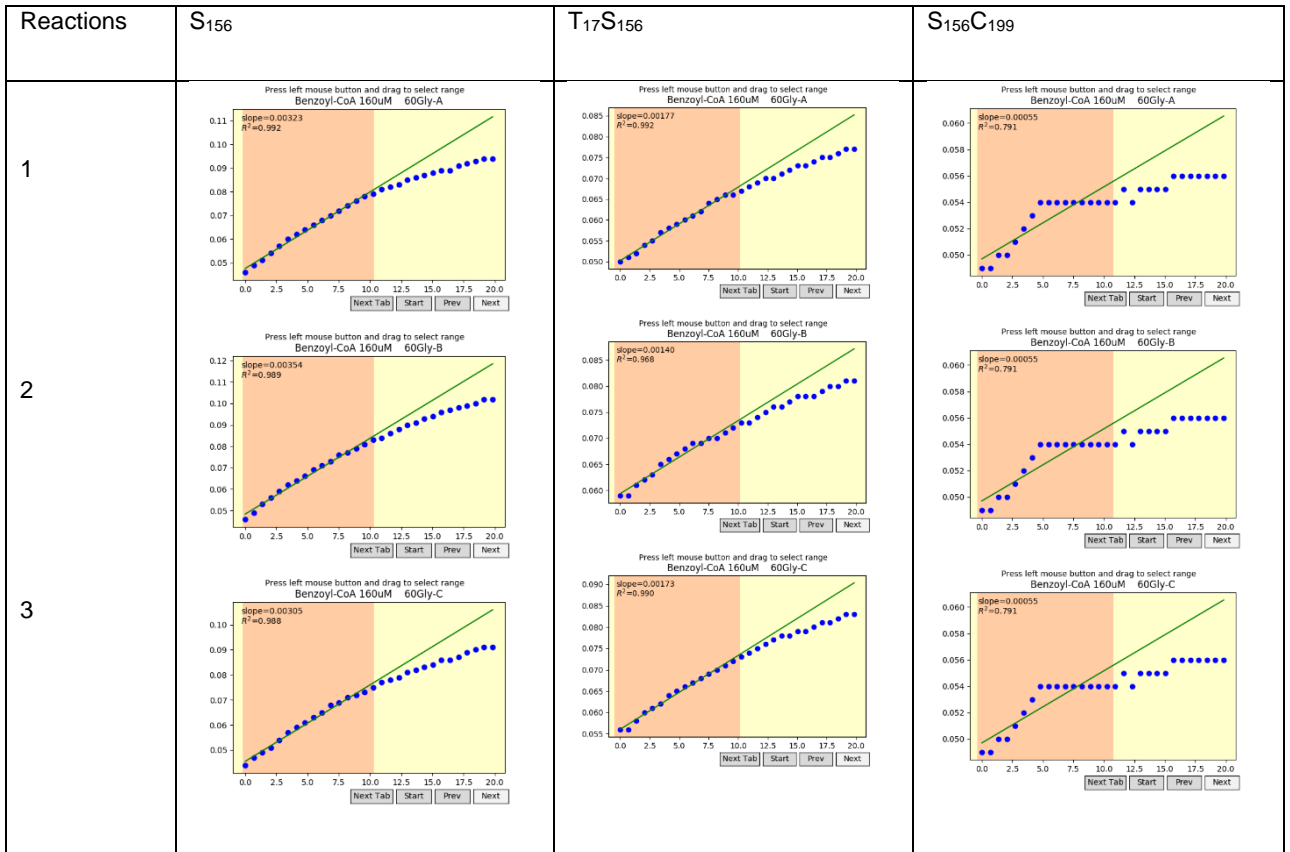
## Benzoyl-CoA : 160μM and Glycine 20mM



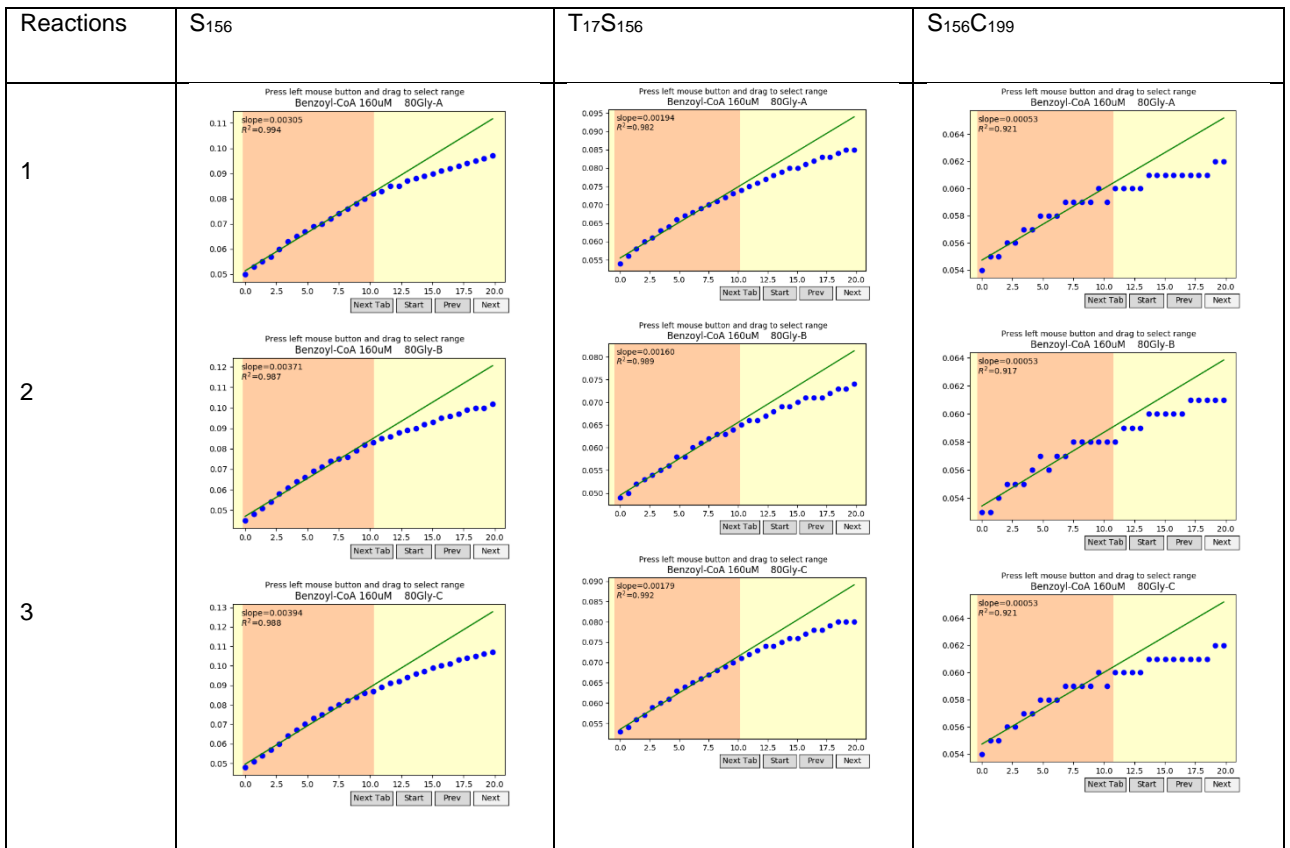
## Benzoyl-CoA : 160μM and Glycine 40mM



## Benzoyl-CoA : 160μM and Glycine 60mM



## Benzoyl-CoA : 160μM and Glycine 80mM



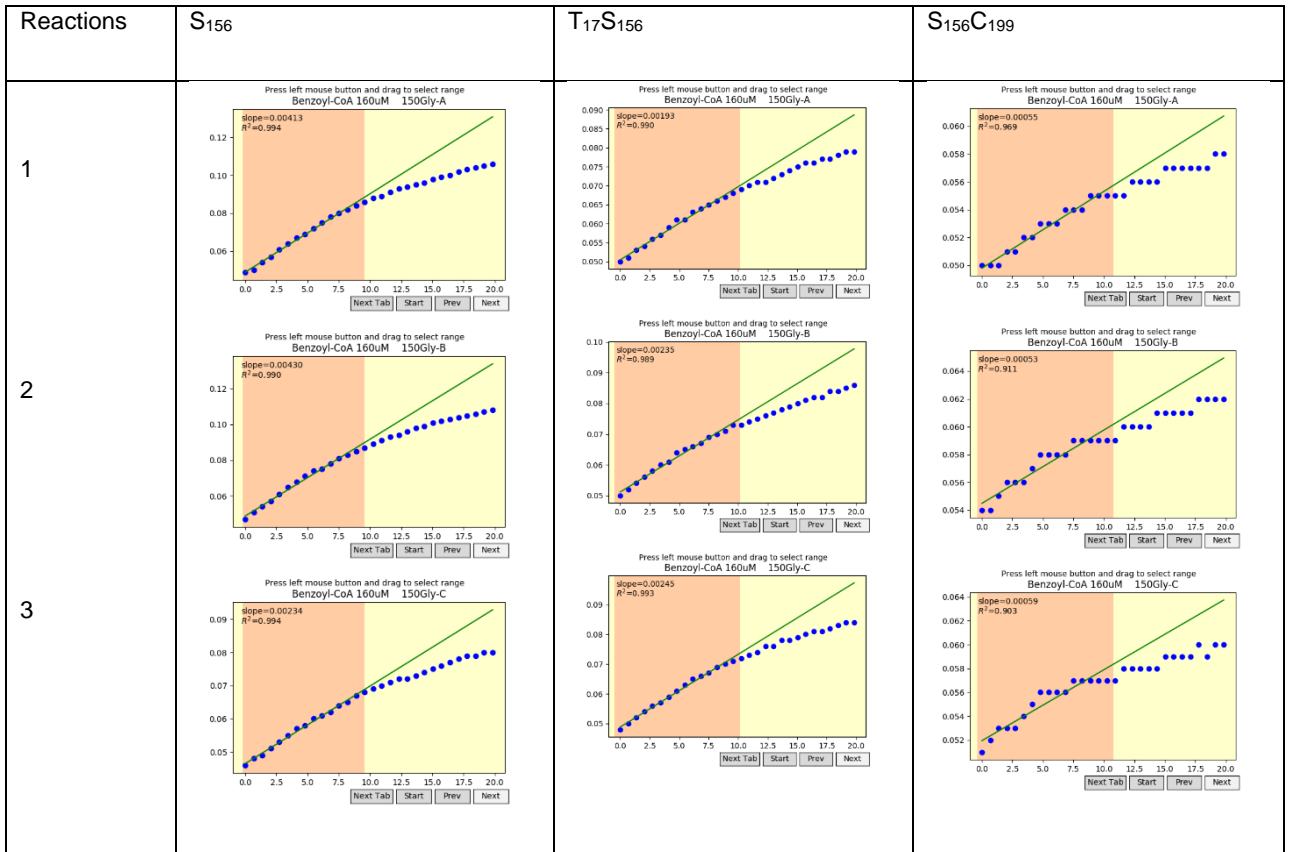
**Benzoyl-CoA : 160µM and Glycine 100mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 100Gly-C</p>

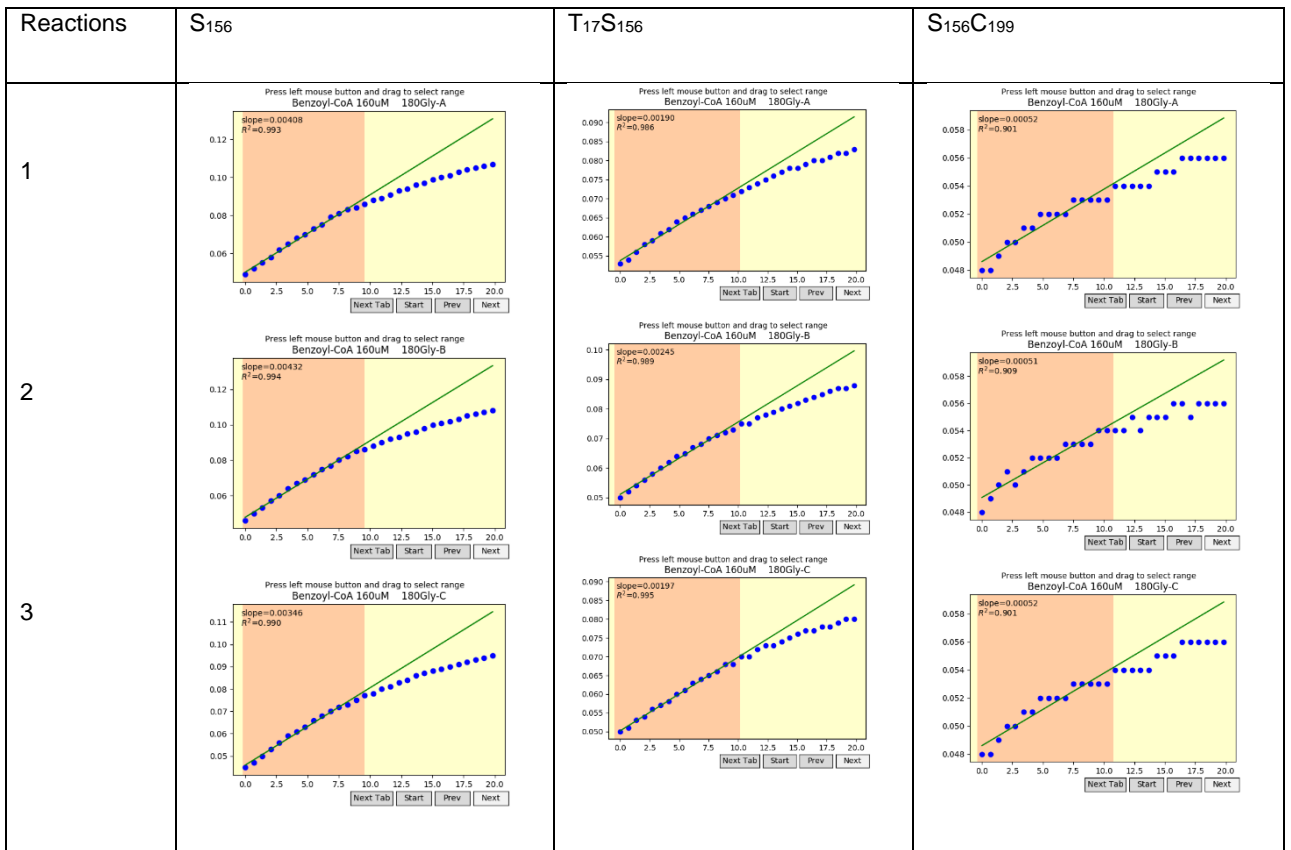
**Benzoyl-CoA : 160µM and Glycine 120mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-A</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-A</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-B</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-B</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-C</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160µM 120Gly-C</p>

**Benzoyl-CoA : 160μM and Glycine 150mM**



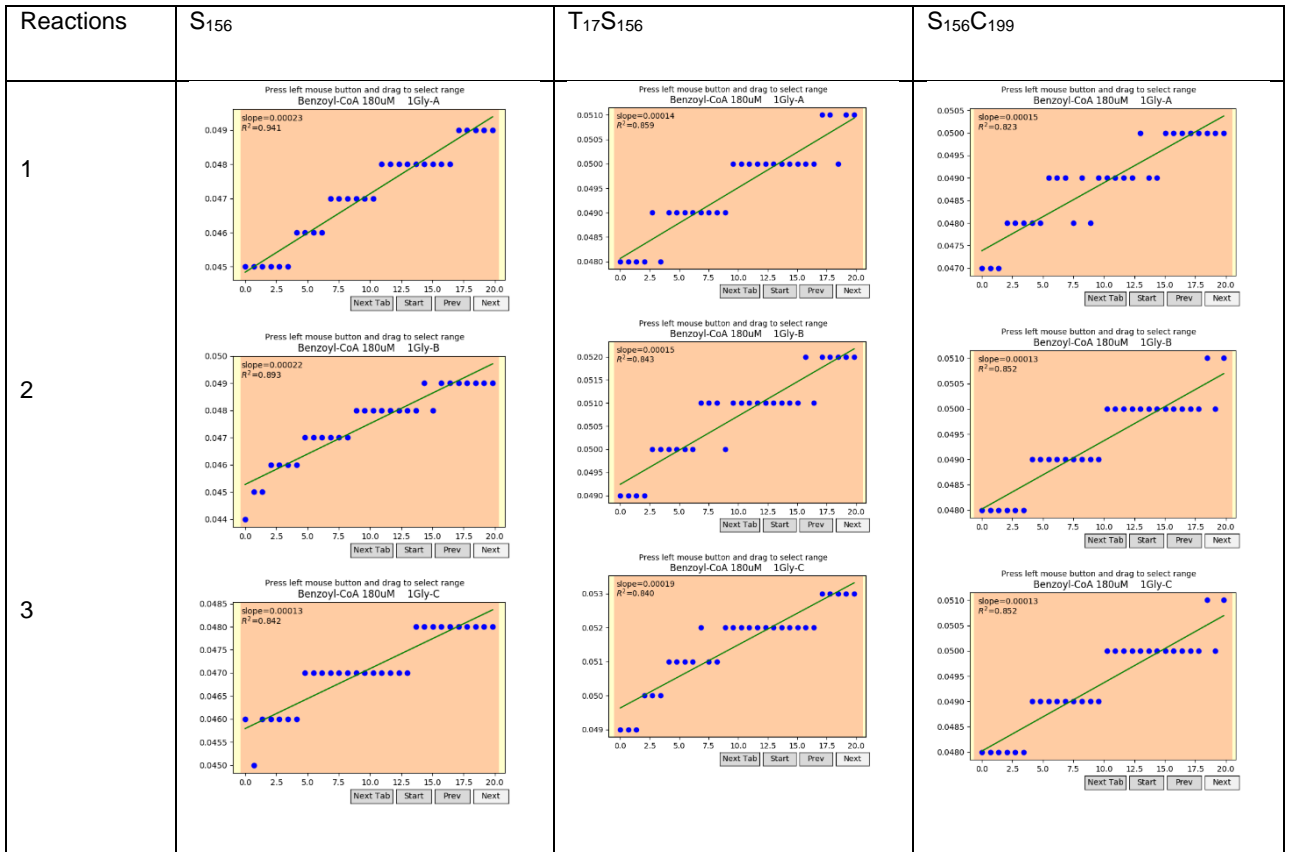
**Benzoyl-CoA : 160μM and Glycine 180mM**



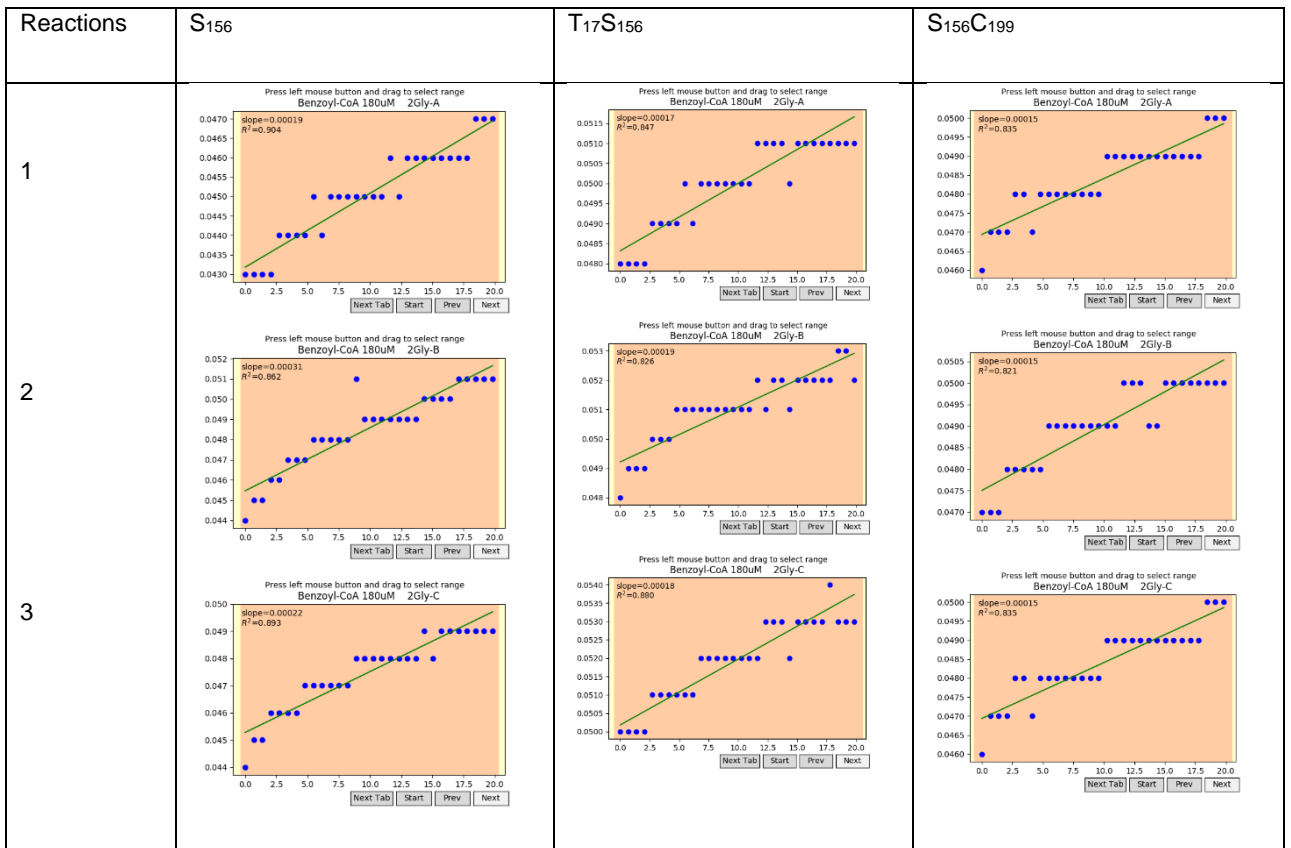
**Benzoyl-CoA : 160 $\mu$ M and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-A</p> <p>slope=0.00389 R<sup>2</sup>=0.993</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-A</p> <p>slope=0.00186 R<sup>2</sup>=0.993</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-A</p> <p>slope=0.00039 R<sup>2</sup>=0.894</p> <p>Next Tab Start Prev Next</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-B</p> <p>slope=0.00452 R<sup>2</sup>=0.986</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-B</p> <p>slope=0.00239 R<sup>2</sup>=0.996</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-B</p> <p>slope=0.00056 R<sup>2</sup>=0.906</p> <p>Next Tab Start Prev Next</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-C</p> <p>slope=0.00404 R<sup>2</sup>=0.985</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-C</p> <p>slope=0.00242 R<sup>2</sup>=0.989</p> <p>Next Tab Start Prev Next</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 160<math>\mu</math>M 200Gly-C</p> <p>slope=0.00039 R<sup>2</sup>=0.894</p> <p>Next Tab Start Prev Next</p>

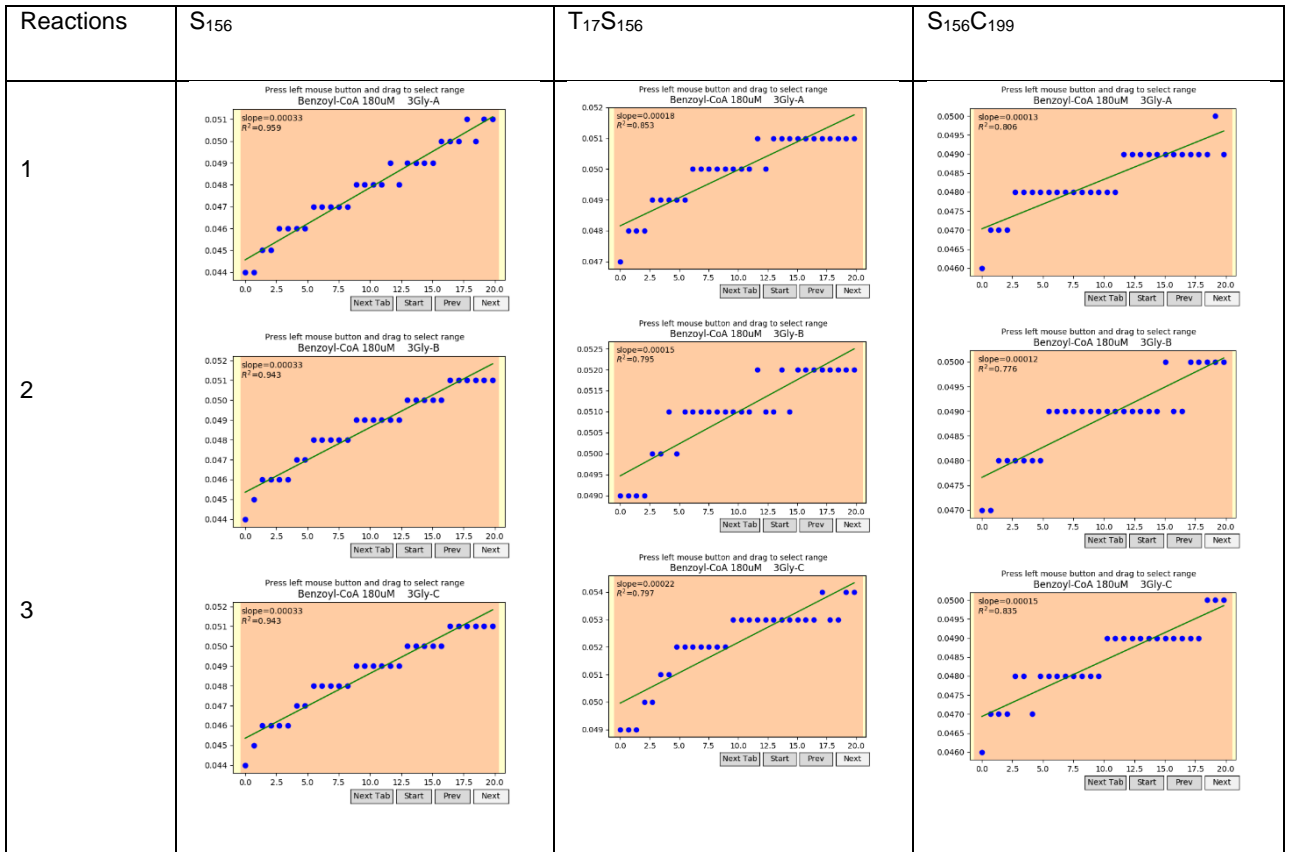
### Benzoyl-CoA : 180µM and Glycine 1mM



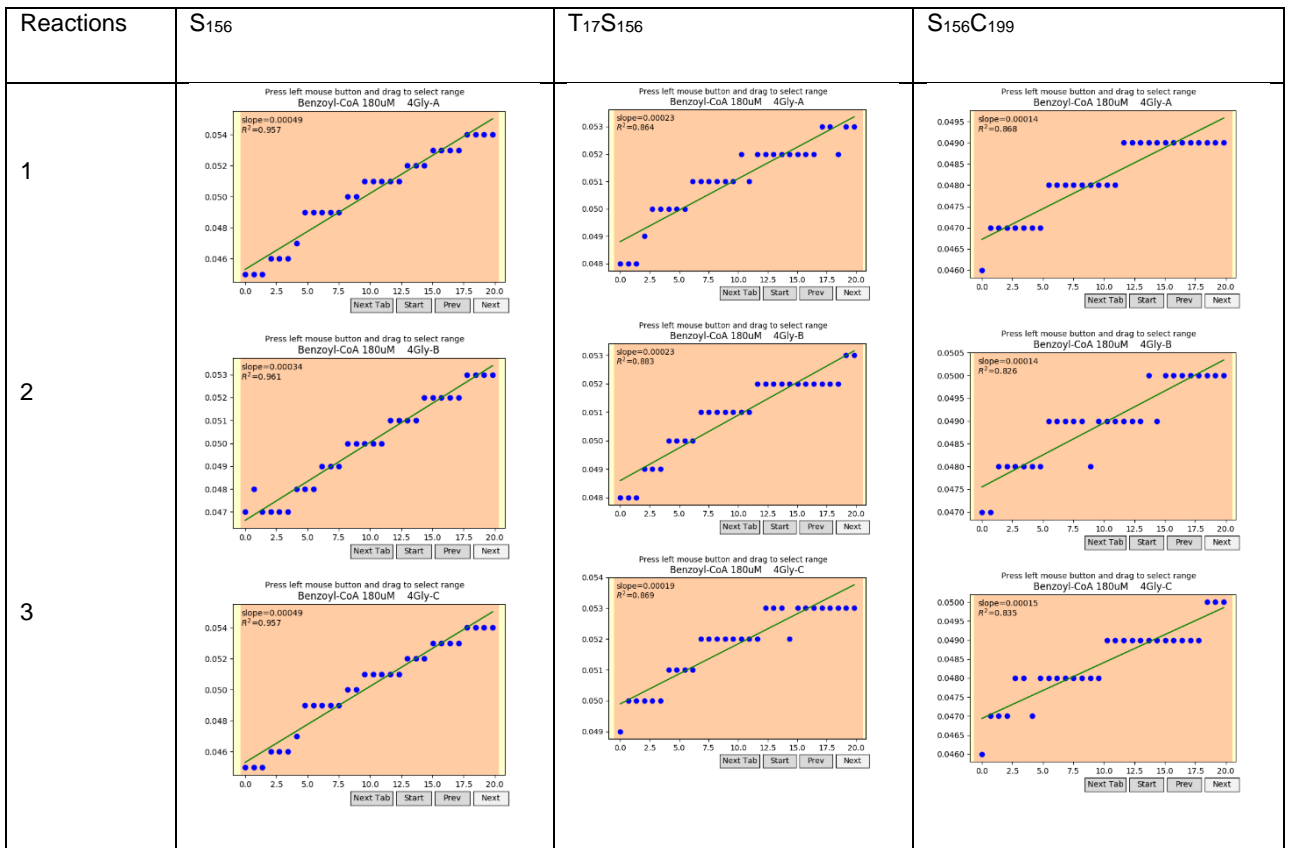
### Benzoyl-CoA : 180µM and Glycine 2mM



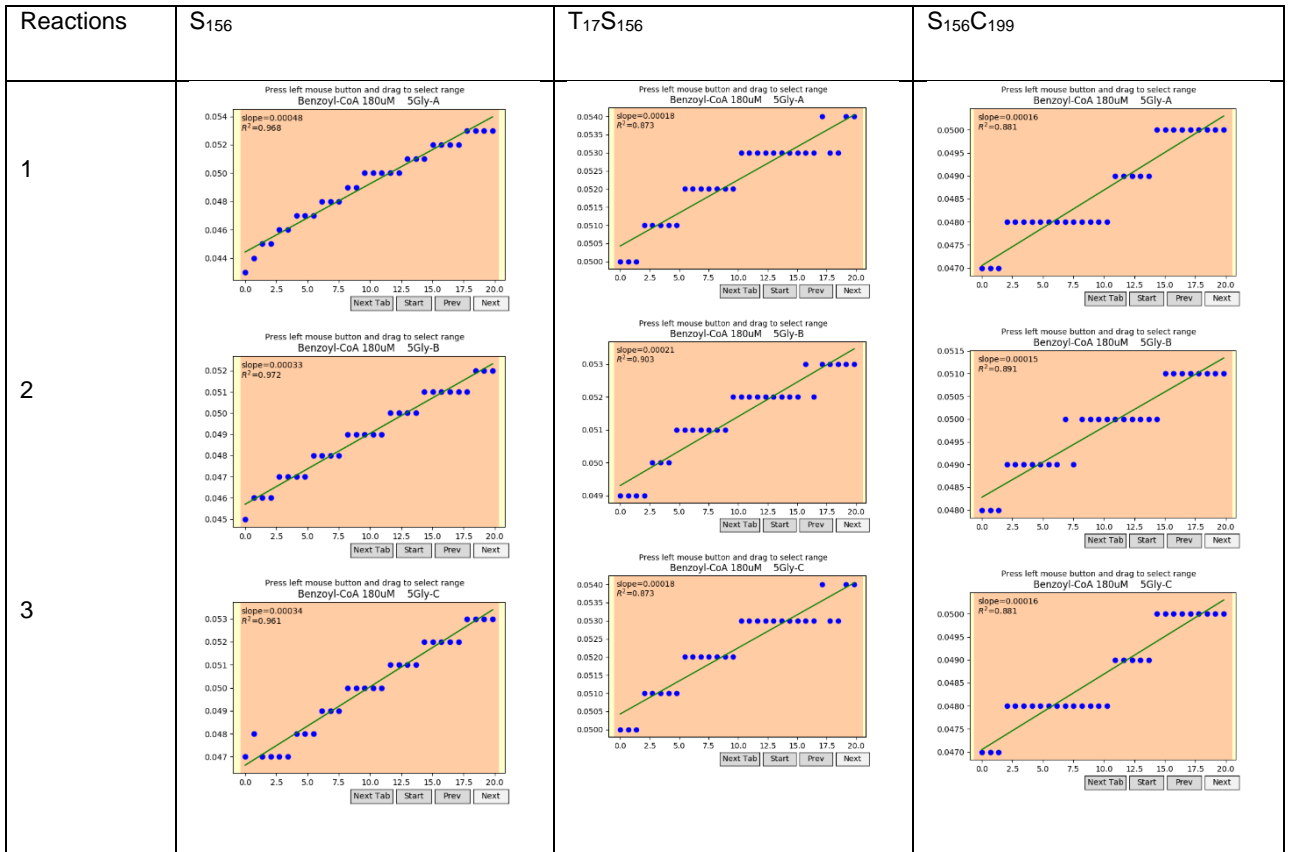
## Benzoyl-CoA : 180µM and Glycine 3mM



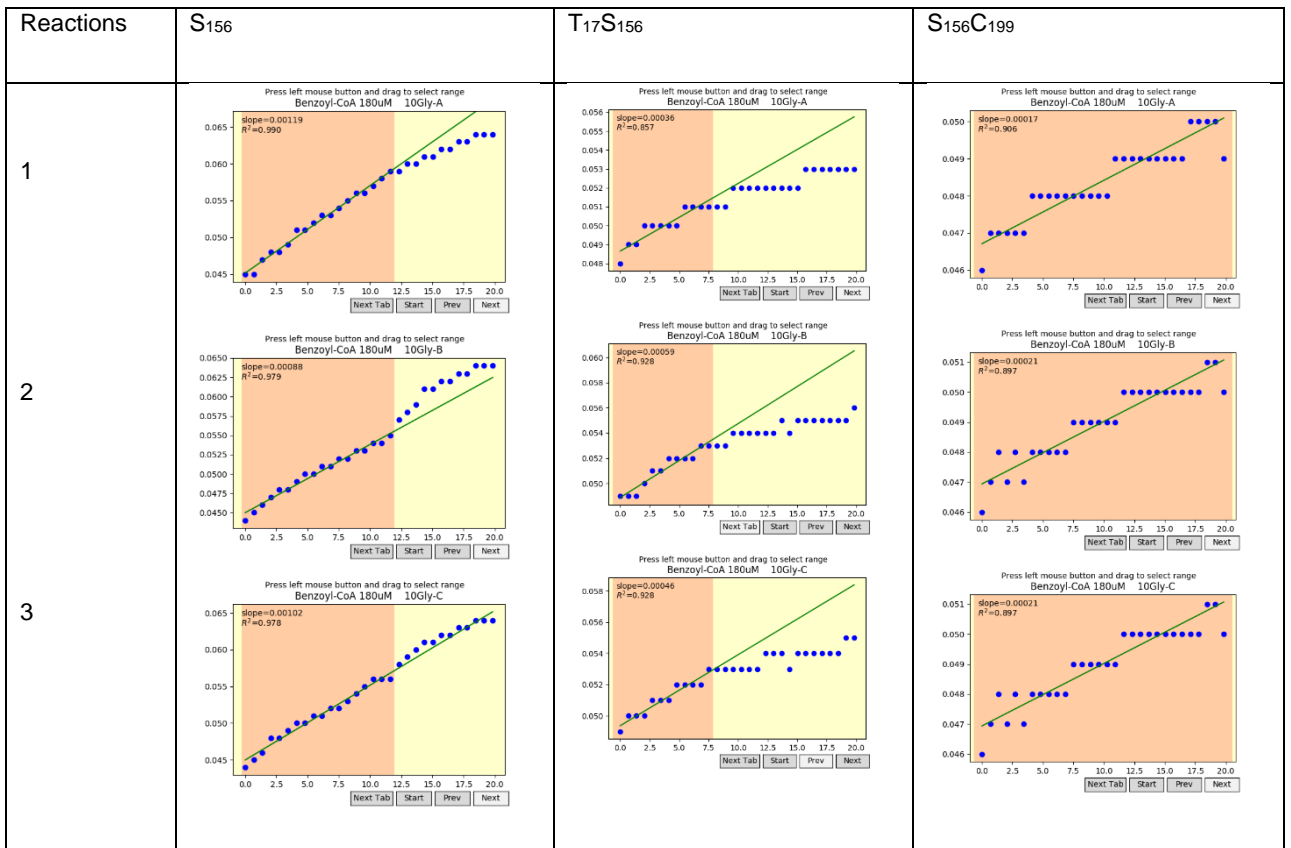
## Benzoyl-CoA : 180µM and Glycine 4mM



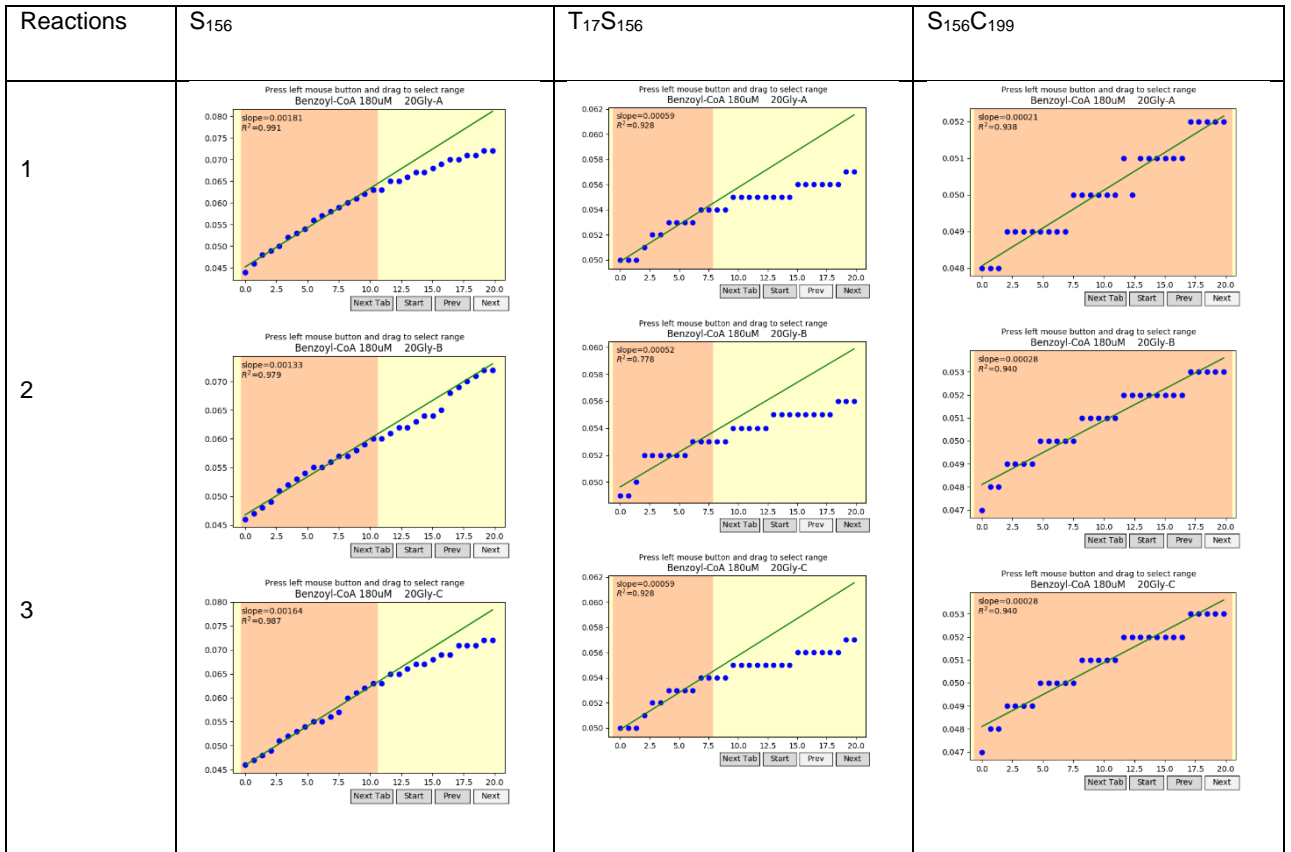
## Benzoyl-CoA : 180µM and Glycine 5mM



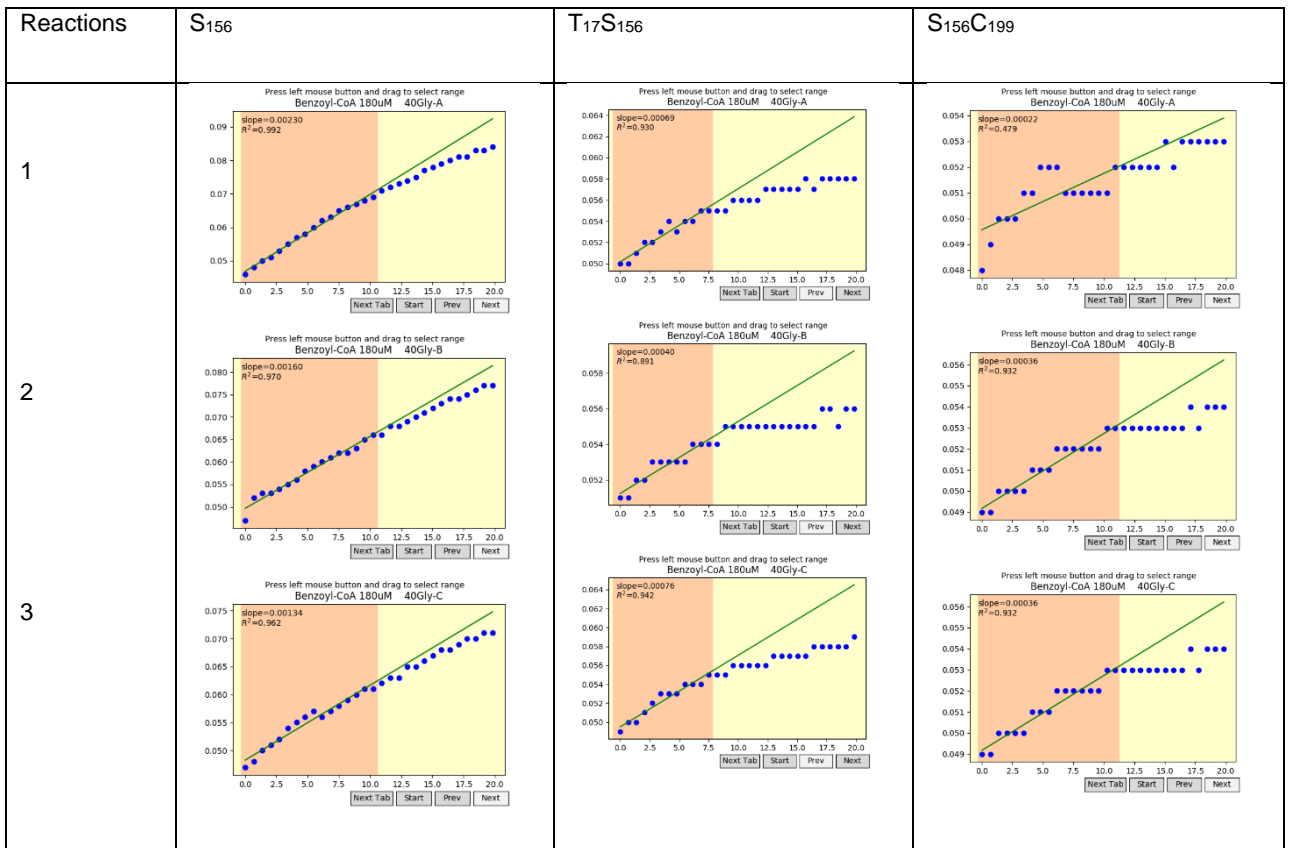
## Benzoyl-CoA : 180µM and Glycine 10mM



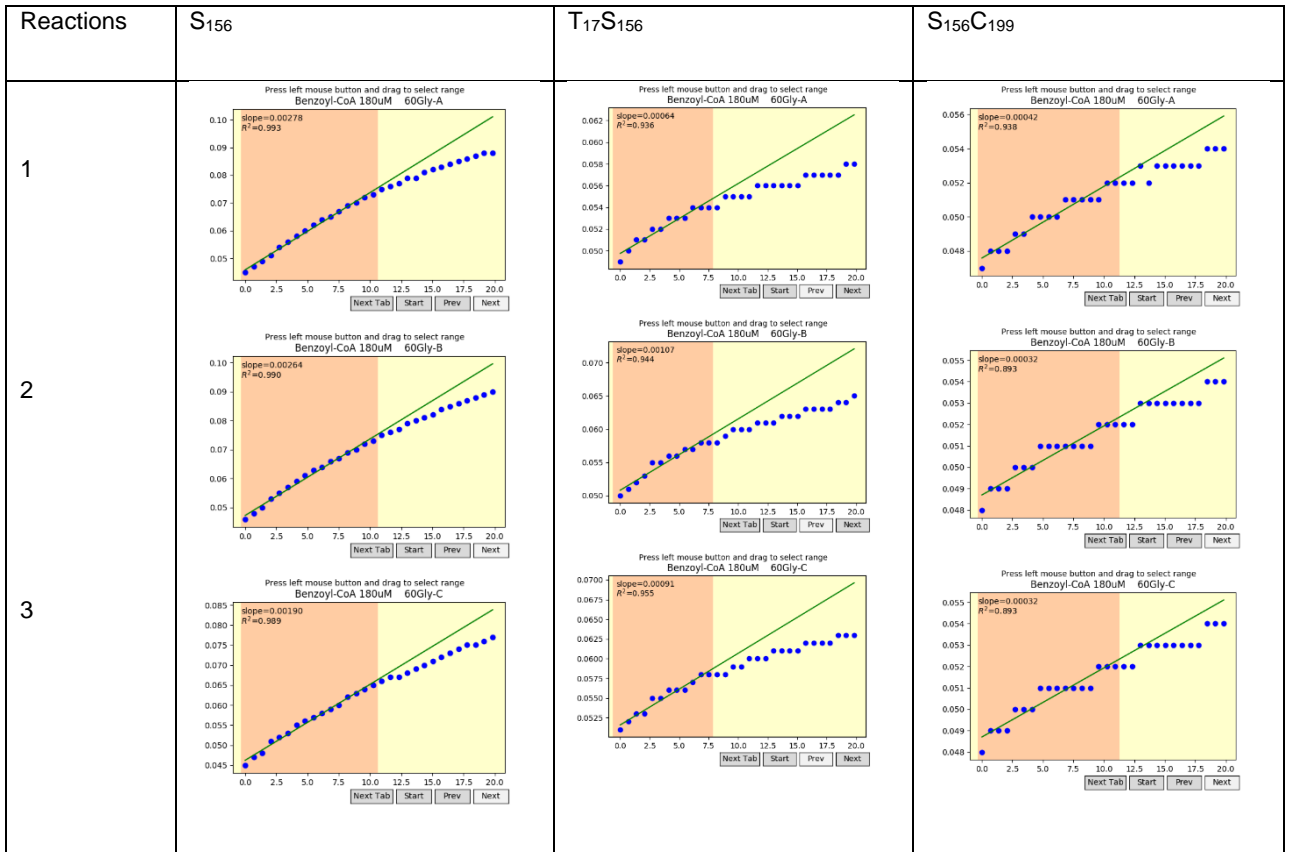
## Benzoyl-CoA : 180µM and Glycine 20mM



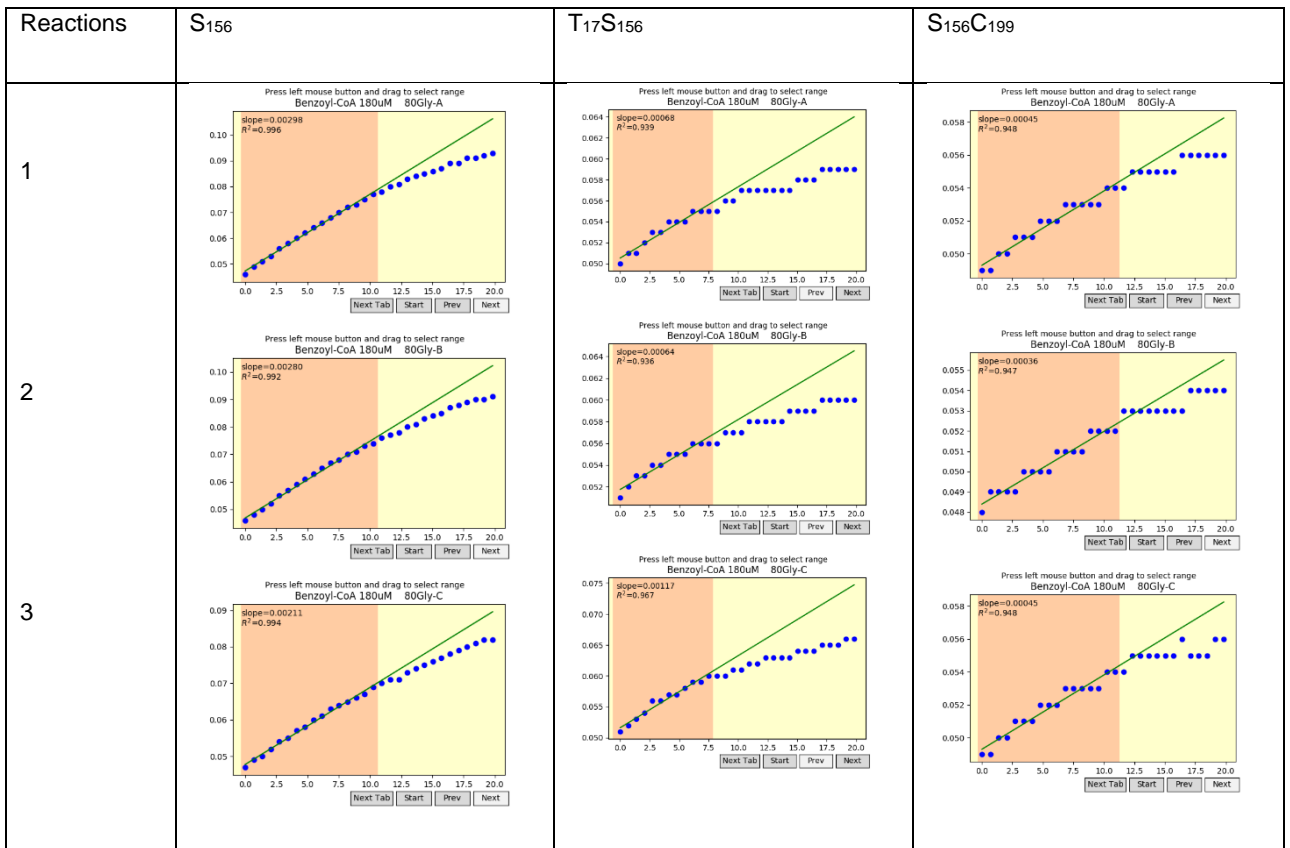
## Benzoyl-CoA : 180µM and Glycine 40mM



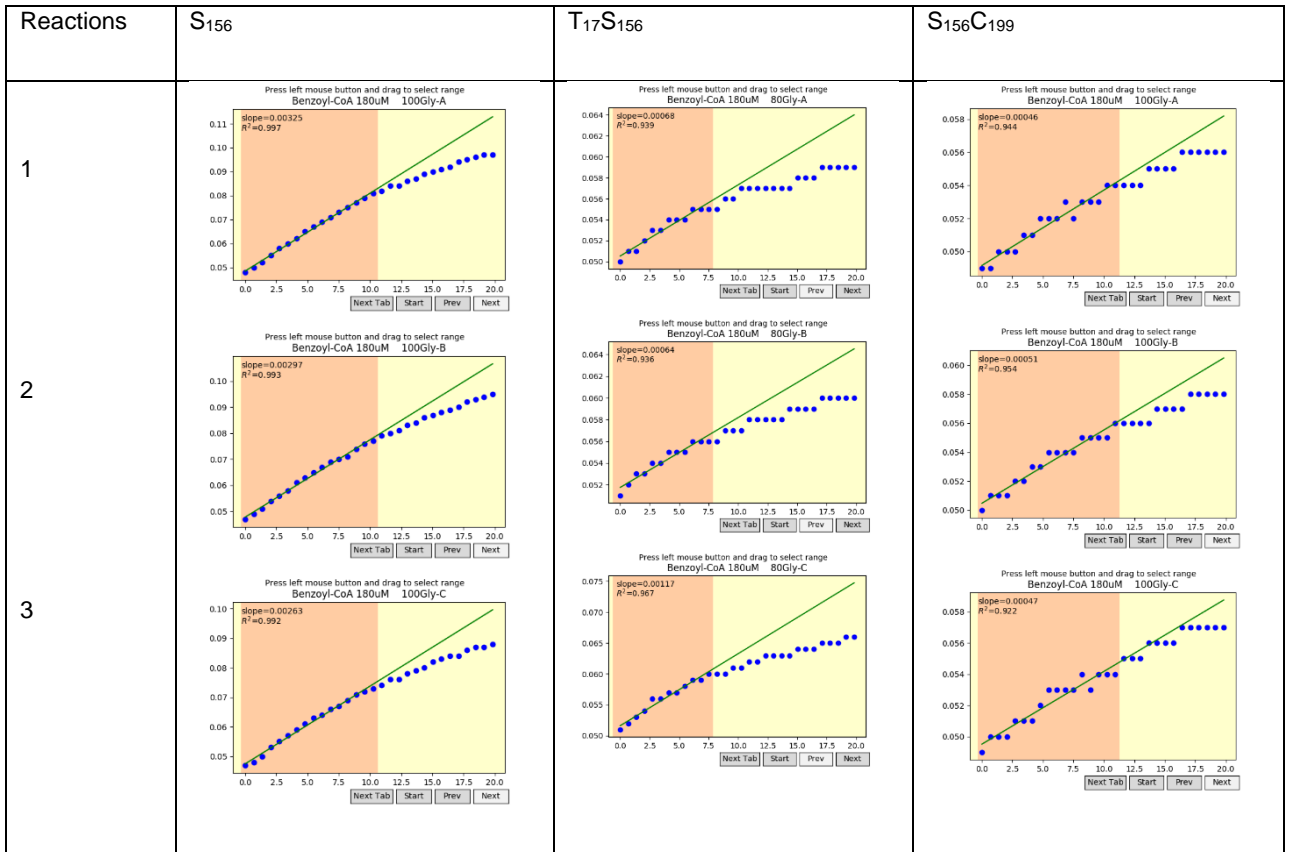
## Benzoyl-CoA : 180μM and Glycine 60mM



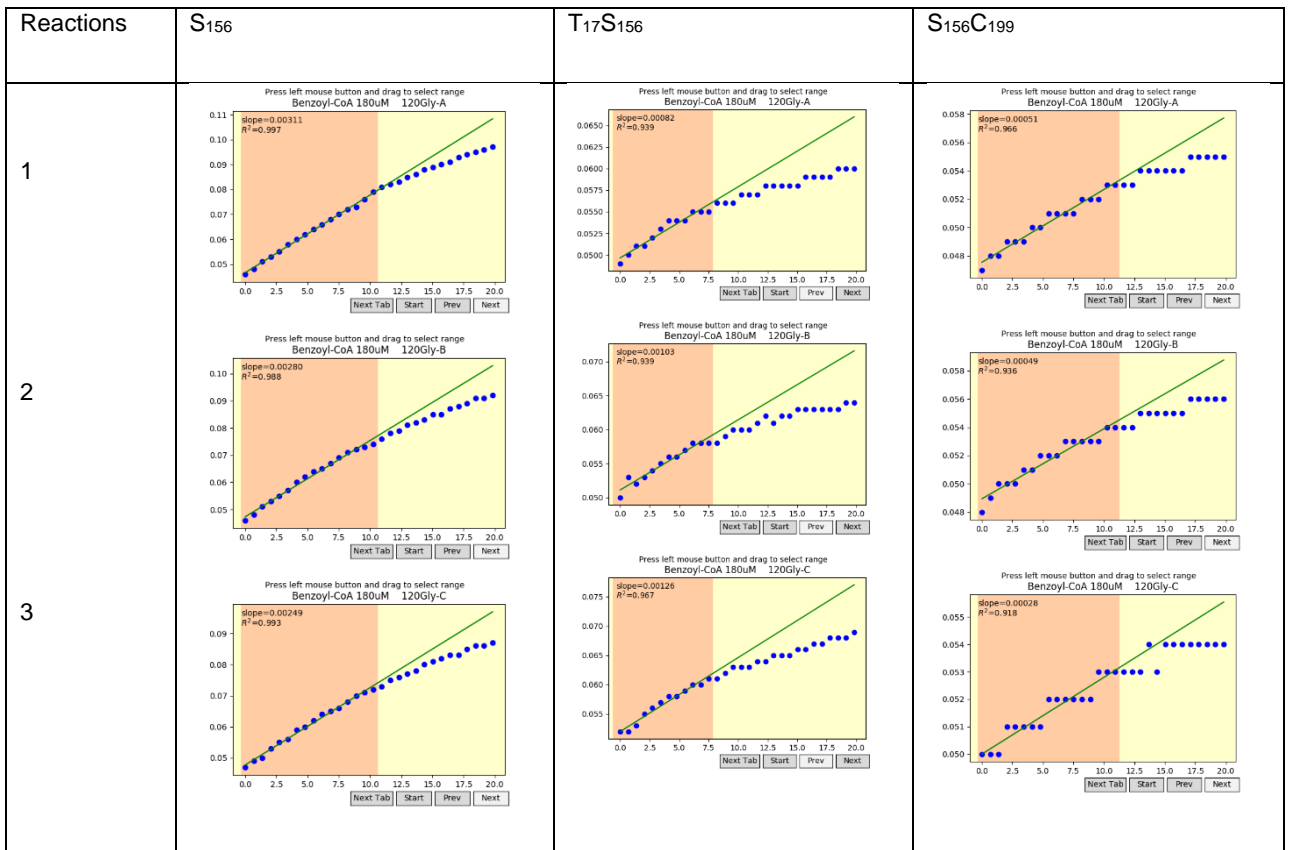
## Benzoyl-CoA : 180μM and Glycine 80mM



**Benzoyl-CoA : 180µM and Glycine 100mM**



**Benzoyl-CoA : 180µM and Glycine 120mM**



**Benzoyl-CoA : 180μM and Glycine 150mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-A</p> <p>slope=0.00360 R<sup>2</sup>=0.993</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-A</p> <p>slope=0.00086 R<sup>2</sup>=0.944</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-A</p> <p>slope=0.00048 R<sup>2</sup>=0.962</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-B</p> <p>slope=0.00287 R<sup>2</sup>=0.999</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-B</p> <p>slope=0.00106 R<sup>2</sup>=0.974</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-B</p> <p>slope=0.00049 R<sup>2</sup>=0.963</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-C</p> <p>slope=0.00274 R<sup>2</sup>=0.994</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-C</p> <p>slope=0.00099 R<sup>2</sup>=0.945</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 150Gly-C</p> <p>slope=0.00044 R<sup>2</sup>=0.953</p>

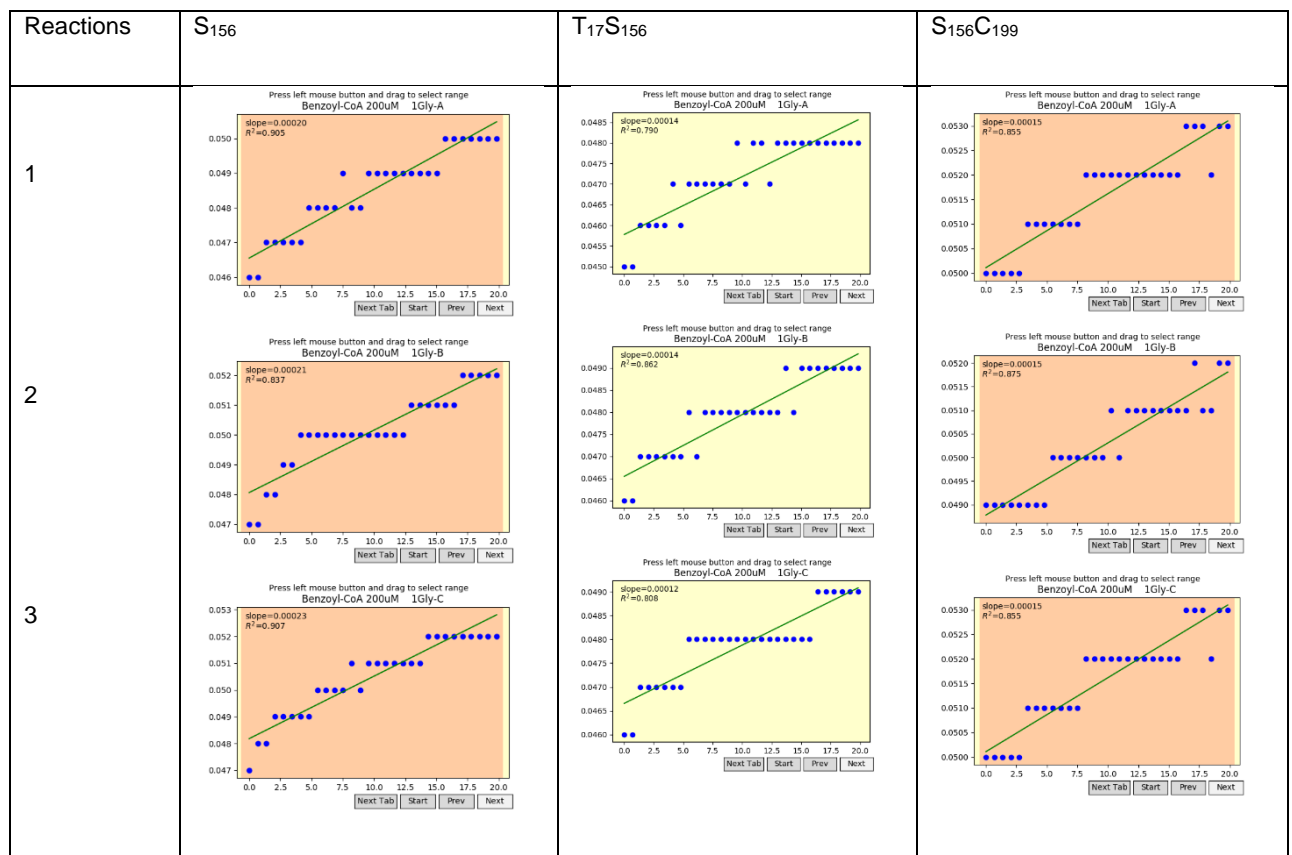
**Benzoyl-CoA : 180μM and Glycine 180mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-A</p> <p>slope=0.00356 R<sup>2</sup>=0.991</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-A</p> <p>slope=0.00062 R<sup>2</sup>=0.908</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-A</p> <p>slope=0.00054 R<sup>2</sup>=0.956</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-B</p> <p>slope=0.00297 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-B</p> <p>slope=0.00116 R<sup>2</sup>=0.953</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-B</p> <p>slope=0.00047 R<sup>2</sup>=0.922</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-C</p> <p>slope=0.00319 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-C</p> <p>slope=0.00142 R<sup>2</sup>=0.956</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180μM 180Gly-C</p> <p>slope=0.00029 R<sup>2</sup>=0.834</p>

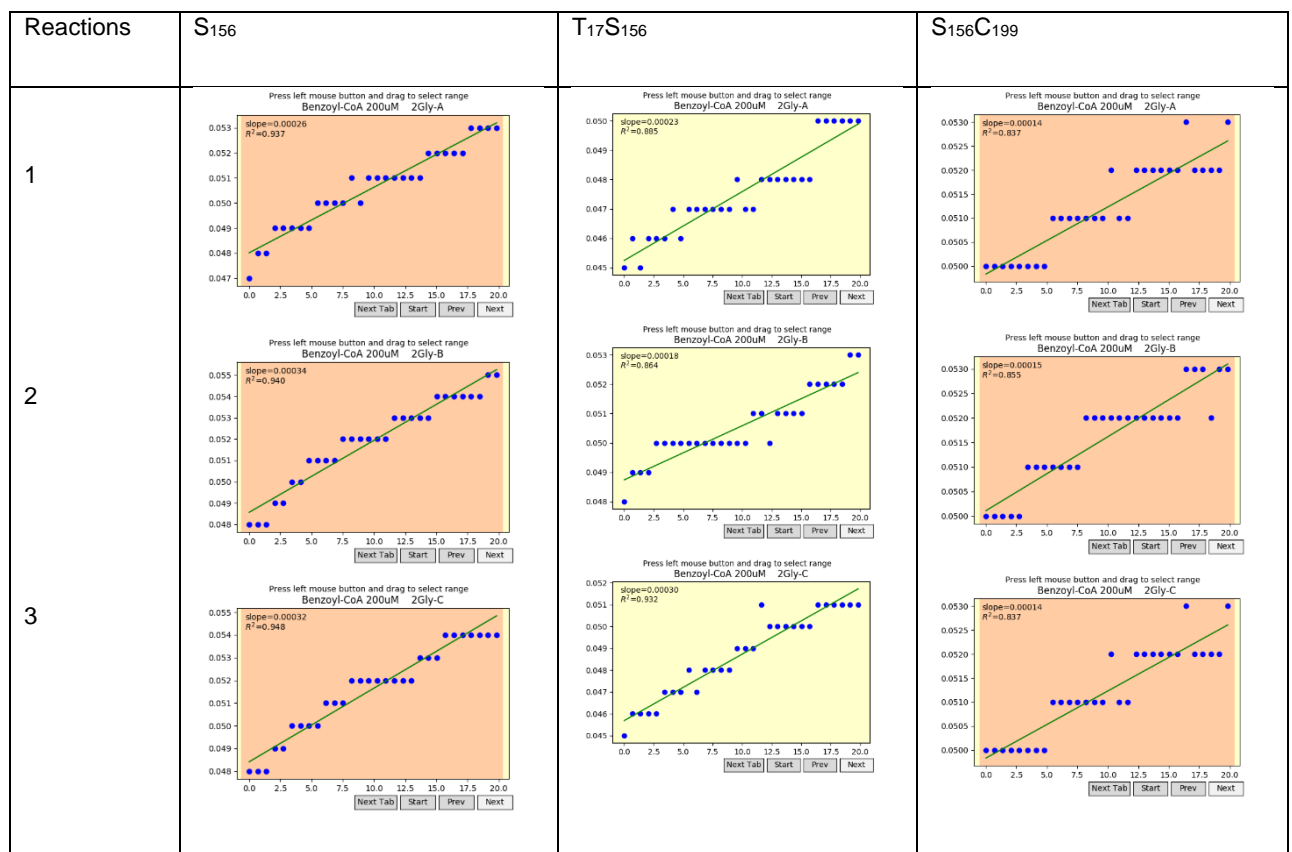
**Benzoyl-CoA : 180 $\mu$ M and Glycine 200mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-A</p> <p>slope=0.00328 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-A</p> <p>slope=0.00073 R<sup>2</sup>=0.967</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-A</p> <p>slope=0.00049 R<sup>2</sup>=0.963</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-B</p> <p>slope=0.00290 R<sup>2</sup>=0.996</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-B</p> <p>slope=0.00126 R<sup>2</sup>=0.974</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-B</p> <p>slope=0.00047 R<sup>2</sup>=0.960</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-C</p> <p>slope=0.00345 R<sup>2</sup>=0.996</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-C</p> <p>slope=0.00146 R<sup>2</sup>=0.952</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 180<math>\mu</math>M 200Gly-C</p> <p>slope=0.00047 R<sup>2</sup>=0.960</p>

## Benzoyl-CoA : 200μM and Glycine 1mM



## Benzoyl-CoA : 200μM and Glycine 2mM



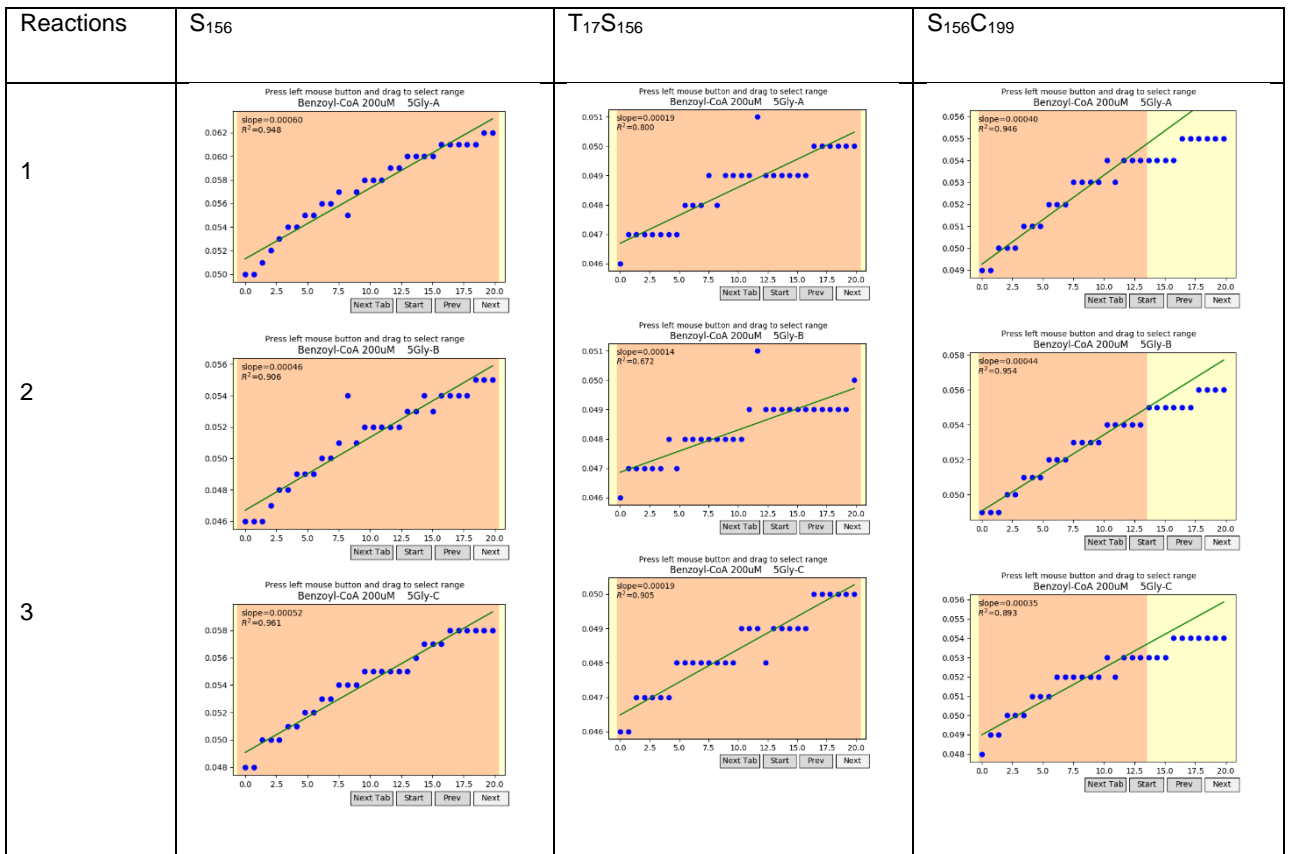
**Benzoyl-CoA : 200µM and Glycine 3mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-A slope=0.00035 R<sup>2</sup>=0.919</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-A slope=0.00017 R<sup>2</sup>=0.848</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-A slope=0.00010 R<sup>2</sup>=0.960</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-B slope=0.00030 R<sup>2</sup>=0.884</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-B slope=0.00015 R<sup>2</sup>=0.661</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-B slope=0.00014 R<sup>2</sup>=0.743</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-C slope=0.00034 R<sup>2</sup>=0.940</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-C slope=0.00015 R<sup>2</sup>=0.858</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 3Gly-C slope=0.00015 R<sup>2</sup>=0.875</p>

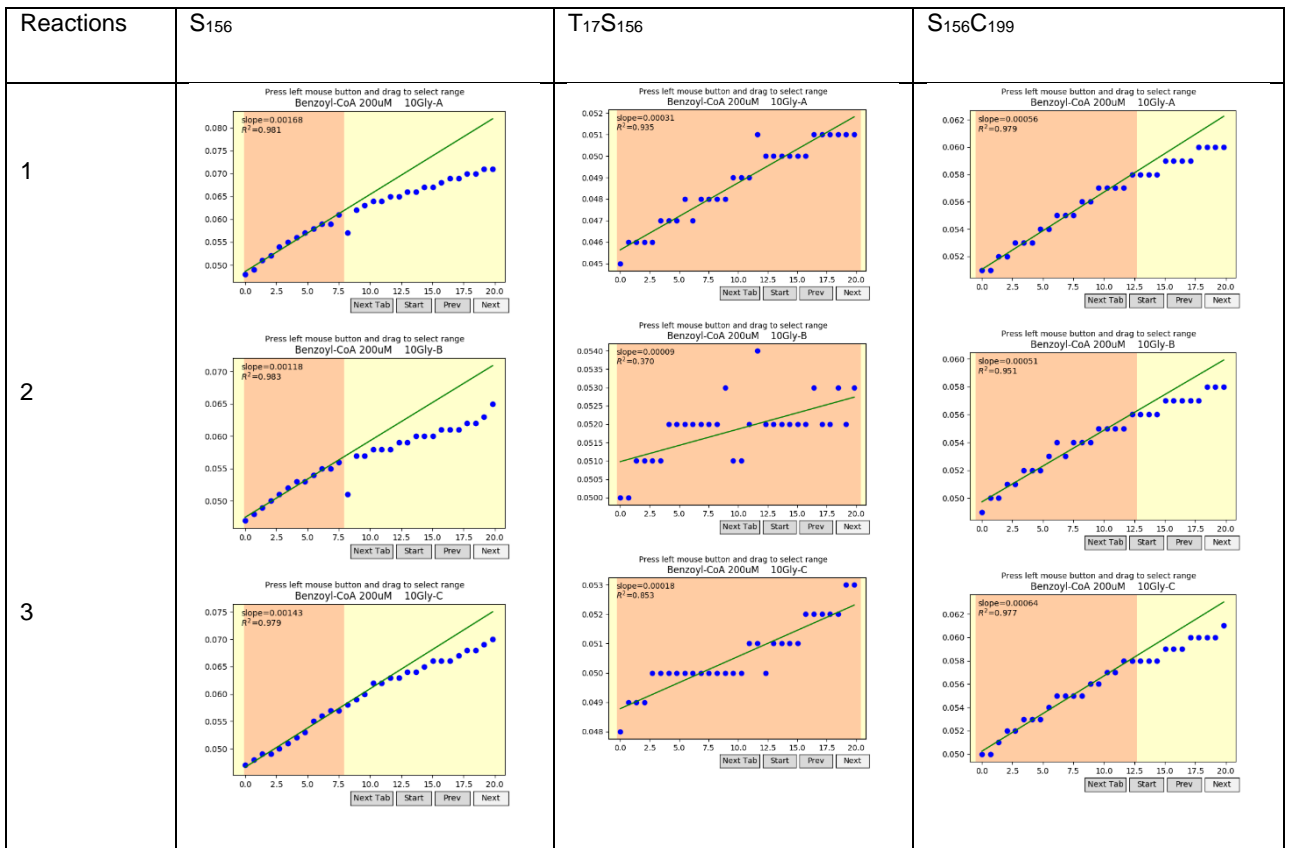
**Benzoyl-CoA : 200µM and Glycine 4mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-A slope=0.00037 R<sup>2</sup>=0.942</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-A slope=0.00016 R<sup>2</sup>=0.858</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-A slope=0.00021 R<sup>2</sup>=0.911</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-B slope=0.00045 R<sup>2</sup>=0.954</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-B slope=0.00019 R<sup>2</sup>=0.905</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-B slope=0.00017 R<sup>2</sup>=0.847</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-C slope=0.00045 R<sup>2</sup>=0.954</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-C slope=0.00016 R<sup>2</sup>=0.833</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 4Gly-C slope=0.00017 R<sup>2</sup>=0.847</p>

## Benzoyl-CoA : 200µM and Glycine 5mM



## Benzoyl-CoA : 200µM and Glycine 10mM



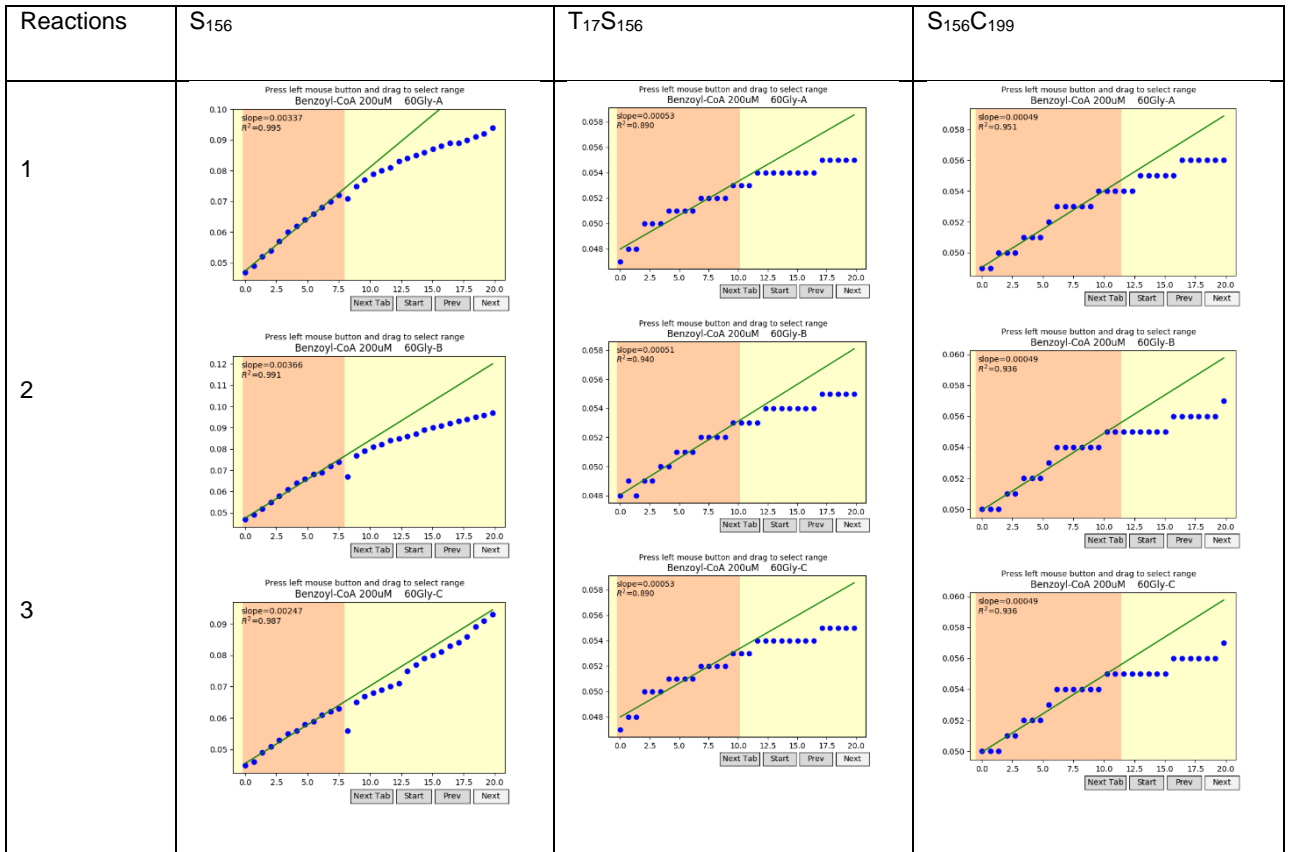
**Benzoyl-CoA : 200 $\mu$ M and Glycine 20mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-A</p> <p>slope=0.00215 R<sup>2</sup>=0.982</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-A</p> <p>slope=0.00041 R<sup>2</sup>=0.939</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-A</p> <p>slope=0.00048 R<sup>2</sup>=0.966</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-B</p> <p>slope=0.00157 R<sup>2</sup>=0.987</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-B</p> <p>slope=0.00034 R<sup>2</sup>=0.877</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-B</p> <p>slope=0.00038 R<sup>2</sup>=0.873</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-C</p> <p>slope=0.00202 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-C</p> <p>slope=0.00034 R<sup>2</sup>=0.891</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 20Gly-C</p> <p>slope=0.00043 R<sup>2</sup>=0.939</p>

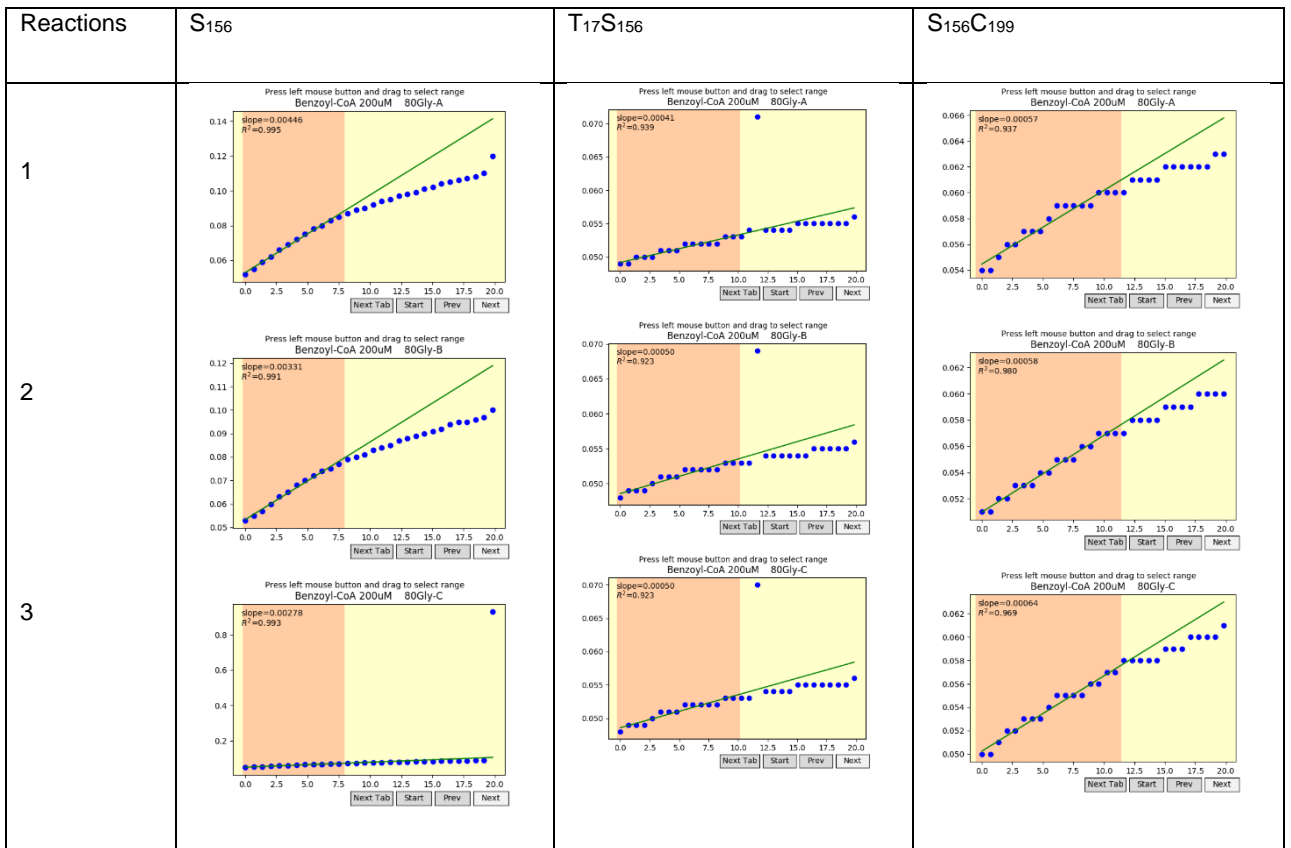
**Benzoyl-CoA : 200 $\mu$ M and Glycine 40mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 40Gly-A</p> <p>slope=0.00318 R<sup>2</sup>=0.985</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 60Gly-A</p> <p>slope=0.00053 R<sup>2</sup>=0.890</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 40Gly-A</p> <p>slope=0.00044 R<sup>2</sup>=0.912</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 40Gly-B</p> <p>slope=0.00272 R<sup>2</sup>=0.981</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 60Gly-B</p> <p>slope=0.00051 R<sup>2</sup>=0.840</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 40Gly-B</p> <p>slope=0.00043 R<sup>2</sup>=0.939</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 40Gly-C</p> <p>slope=0.00314 R<sup>2</sup>=0.997</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 60Gly-C</p> <p>slope=0.00053 R<sup>2</sup>=0.890</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 40Gly-C</p> <p>slope=0.00043 R<sup>2</sup>=0.939</p>

## Benzoyl-CoA : 200μM and Glycine 60mM



## Benzoyl-CoA : 200μM and Glycine 80mM



**Benzoyl-CoA : 200µM and Glycine 100mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-A</p> <p>slope=0.00357 R<sup>2</sup>=0.992</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-A</p> <p>slope=0.00056 R<sup>2</sup>=0.938</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-A</p> <p>slope=0.00048 R<sup>2</sup>=0.974</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-B</p> <p>slope=0.00407 R<sup>2</sup>=0.989</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-B</p> <p>slope=0.00053 R<sup>2</sup>=0.954</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-B</p> <p>slope=0.00040 R<sup>2</sup>=0.951</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-C</p> <p>slope=0.00365 R<sup>2</sup>=0.997</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-C</p> <p>slope=0.00059 R<sup>2</sup>=0.972</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 100Gly-C</p> <p>slope=0.00059 R<sup>2</sup>=0.976</p>

**Benzoyl-CoA : 200µM and Glycine 120mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-A</p> <p>slope=0.00293 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-A</p> <p>slope=0.00057 R<sup>2</sup>=0.956</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-A</p> <p>slope=0.00059 R<sup>2</sup>=0.976</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-B</p> <p>slope=0.00433 R<sup>2</sup>=0.990</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-B</p> <p>slope=0.00044 R<sup>2</sup>=0.936</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-B</p> <p>slope=0.00037 R<sup>2</sup>=0.912</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-C</p> <p>slope=0.00407 R<sup>2</sup>=0.993</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-C</p> <p>slope=0.00059 R<sup>2</sup>=0.972</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200µM 120Gly-C</p> <p>slope=0.00055 R<sup>2</sup>=0.968</p>

**Benzoyl-CoA : 200μM and Glycine 150mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-A</p> <p>slope=0.00492 R<sup>2</sup>=0.994</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-A</p> <p>slope=0.00064 R<sup>2</sup>=0.968</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-A</p> <p>slope=0.00055 R<sup>2</sup>=0.968</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-B</p> <p>slope=0.00428 R<sup>2</sup>=0.986</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-B</p> <p>slope=0.00056 R<sup>2</sup>=0.953</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-B</p> <p>slope=0.00047 R<sup>2</sup>=0.956</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-C</p> <p>slope=0.00342 R<sup>2</sup>=0.992</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-C</p> <p>slope=0.00053 R<sup>2</sup>=0.890</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 150Gly-C</p> <p>slope=0.00059 R<sup>2</sup>=0.976</p>

**Benzoyl-CoA : 200μM and Glycine 180mM**

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-A</p> <p>slope=0.00508 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-A</p> <p>slope=0.00041 R<sup>2</sup>=0.939</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-A</p> <p>slope=0.00060 R<sup>2</sup>=0.956</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-B</p> <p>slope=0.00355 R<sup>2</sup>=0.989</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-B</p> <p>slope=0.00048 R<sup>2</sup>=0.953</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-B</p> <p>slope=0.00042 R<sup>2</sup>=0.913</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-C</p> <p>slope=0.00451 R<sup>2</sup>=0.989</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-C</p> <p>slope=0.00064 R<sup>2</sup>=0.968</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200uM 180Gly-C</p> <p>slope=0.00049 R<sup>2</sup>=0.936</p>

# Benzoyl-CoA : 200 $\mu$ M and Glycine 200mM

Reactions	S <sub>156</sub>	T <sub>17</sub> S <sub>156</sub>	S <sub>156</sub> C <sub>199</sub>
1	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-A</p> <p>slope=0.00469 R<sup>2</sup>=0.994</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-A</p> <p>slope=0.00048 R<sup>2</sup>=0.953</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-A</p> <p>slope=0.00055 R<sup>2</sup>=0.972</p>
2	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-B</p> <p>slope=0.00308 R<sup>2</sup>=0.995</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-B</p> <p>slope=0.00044 R<sup>2</sup>=0.958</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-B</p> <p>slope=0.00011 R<sup>2</sup>=0.418</p>
3	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-C</p> <p>slope=0.00315 R<sup>2</sup>=0.998</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-C</p> <p>slope=0.00015 R<sup>2</sup>=0.563</p>	<p>Press left mouse button and drag to select range Benzoyl-CoA 200<math>\mu</math>M 200Gly-C</p> <p>slope=0.00043 R<sup>2</sup>=0.939</p>





























