

## Supplementary Information

### Dissecting how modular polyketide synthase ketoreductases interact with acyl carrier protein-attached substrates

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## 1. Materials, DNA isolation and manipulation

Restriction endonucleases and Taq ligase were obtained from New England BioLabs. Phusion DNA polymerase was purchased from Thermo Scientific. T5 exonuclease was purchased from Epicentre. T4DNA ligase, alkaline phosphatase and protease inhibitors were purchased from Roche. All other chemicals were from Sigma-Aldrich.

All constructs were cloned from the *MLSA1* or *MLSB* genes using either transfer polymerase chain reactions [Erijman et al., 2011; Erijman et al., 2014] or Gibson assembly [Gibson et al., 2009] and the primers described in Table S1. The products were treated with *DpnI* for 2 h at 37 °C and transformed into competent *E. coli* DH5 $\alpha$  cells (Life Technologies) prior to conducting colony polymerase chain reactions using the method of Nybo [Nybo, 2012] to filter out false positive colonies. Plasmid DNA was isolated from an overnight culture using a Wizard Mini-prep set (Promega). Final confirmation of cloning steps was obtained by DNA sequencing (DNA Sequencing Facility, Department of Biochemistry, University of Cambridge).

## 2. Protein expression and purification of KR domain samples

### *mKR $\alpha$*

The sequence coding for mKR $\alpha$  in module 5 of the *mlsA1* gene from *Mycobacterium ulcerans* (Uniprot: Q6MZA4; residues 10604-11078; Table S2) was cloned using the primers into pVB, a modified pET28 $\alpha$  vector in which the amino acid recognition sequence for thrombin had been replaced with that for tobacco etch virus (TEV) protease, preceded by an N-terminal His<sub>6</sub>-tag fused to GB1, the 56-residue B1 immunoglobulin binding domain of Streptococcal protein G [Bao et al., 2006]. The pVB-mKR $\alpha$  plasmid was transformed into competent *E. coli* Tuner (DE3) cells (Merck). The His<sub>6</sub>-GB1-mKR $\alpha$  fusion protein was expressed by growing the cells at 37 °C in 1 L of LB medium, prepared according to standard protocols [Sambrook & Russell, 2001], with 30  $\mu$ g/mL kanamycin (Sigma) for selection, to a 600 nm optical density of 0.8, followed by induction with 0.5 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG; Sigma) and incubation at 20 °C for 20 h. Cells were harvested by centrifugation (20 min; 3583 x g), resuspended in lysis buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, pH 8.0) with 5 mM imidazole, 2.5 units/mL benzonase nuclease (EMD Millipore) and Sigmafast EDTA-free protease inhibitor cocktail (Sigma) and then lysed using an Emulsiflex C5 homogeniser (Glen Creston). The clarified lysate was passed through Ni-NTA resin (Qiagen), washed twice with lysis buffer containing 30 mM imidazole and eluted with a buffer containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 300 mM imidazole, 0.01 % (v/v) NaN<sub>3</sub>, pH 8.0. The eluted protein was exchanged into phosphate buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, 0.01 % (v/v) NaN<sub>3</sub>, pH 7.5) and the His<sub>6</sub>-GB1-tag was cleaved by overnight

incubation at 4 °C with 100 µL N-terminally His<sub>6</sub>-tagged TEV protease in 1 mL TEV buffer (1 M Na<sub>2</sub>HPO<sub>4</sub>, 10 mM EDTA, 20 mM DTT, pH 7.5). The released His<sub>6</sub>-GB1-tag was removed by passing the sample through Ni-NTA resin (Qiagen). The resin was flushed with a buffer containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 5 mM imidazole, 0.01 % (v/v) NaN<sub>3</sub>, pH 8.0 and then washed with 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 30 mM imidazole, 0.01 % (v/v) NaN<sub>3</sub>, pH 8.0; all the protein flow through was collected. The His<sub>6</sub>-tagged GB1 and TEV protease were eluted using a buffer containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 30 mM imidazole, 0.01 % (v/v) NaN<sub>3</sub>, pH 8.0. The mKR<sub>a</sub> in the flow through was further purified by size exclusion chromatography using an Äkta Purifier 10 system equipped with a Superdex 75 10/300 column (GE Healthcare) in a buffer containing 25 mM HEPES, 50 mM NaCl. The mKR<sub>a</sub> sample was concentrated using 5000 MWCO Vivaspin 20 columns (Sartorius Stedim) and stored at 4 °C. All expression and purification steps were monitored by SDS-PAGE (NuPAGE) 4-12 % Bis-Tris gels (Life Technologies) stained with InstantBlue (Expedeon) (Figure S2).

#### *mKR<sub>b</sub>*

The sequence coding for mKR<sub>b</sub> in module 7 of the *mlsB* gene from *Mycobacterium ulcerans* (Uniprot: Q32YM8; residues 13784-13874; Table S2) was cloned into pVB and the resulting pVB-mKR<sub>b</sub> plasmid was transformed into competent *E. coli* Tuner (DE3) cells (Merck). The His<sub>6</sub>-GB1-mKR<sub>b</sub> fusion protein was expressed and the released mKR<sub>b</sub> product was purified using the same protocol described above for mKR<sub>a</sub>. All expression and purification steps were monitored by SDS-PAGE (NuPAGE) 4-12 % Bis-Tris gels (Life Technologies) stained with InstantBlue (Expedeon) (Figure S1).

### **3. Protein expression and purification of *apo* ACP samples**

#### *mACP<sub>a</sub>*

The sequence coding for mACP<sub>a</sub> in module 5 of the *MLSA1* gene from *Mycobacterium ulcerans* (Uniprot: Q6MZA4; residues 11087-11185; Table S2) was cloned into pVH, a modified pET28α vector in which the recognition sequence for thrombin had been replaced with that for tobacco etch virus (TEV) protease. The pVH-mACP<sub>a</sub> plasmid was transformed into competent *E. coli* Tuner (DE3) cells (Merck) and N-terminally His<sub>6</sub>-tagged mACP<sub>a</sub> protein was expressed by growing the cells at 37 °C in 1 L of LB medium, prepared according to standard protocols [Sambrook & Russell, 2001], with 30 µg/mL kanamycin (Sigma) for selection, to a 600 nm optical density of 0.8, followed by induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) and incubation at 15 °C for 16 h. Cells were harvested by centrifugation (20 min; 3583 x g), resuspended in lysis buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, pH 8.0) with 5 mM imidazole, 2.5 units/mL benzonase nuclease (EMD Millipore) and Sigmafast EDTA-free protease inhibitor cocktail (Sigma) and lysed using an Emulsiflex

C5 homogeniser (Glen Creston). The clarified lysate was passed through Ni-NTA resin (Qiagen), washed twice with lysis buffer containing 30 mM imidazole and eluted with lysis buffer containing (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 300 mM imidazole, 0.01 % (v/v) NaN<sub>3</sub>, pH 8.0). The eluted protein was exchanged into phosphate buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 7.5) and the His<sub>6</sub>-tag was cleaved by overnight incubation at 4 °C with TEV protease in 1 mL TEV buffer (1 M sodium phosphate, 10 mM EDTA, 20 mM DTT, pH 7.5). The released mACPa was further purified by size exclusion chromatography using an Äkta Purifier 10 system equipped with a Superdex 75 10/300 column (GE Healthcare) in phosphate buffer. The sample was concentrated using 5000 MWCO Vivaspin 20 columns (Sartorius Stedim). All expression and purification steps were monitored by SDS-PAGE (NuPAGE) 4-12 % Bis-Tris gels (Life Technologies) stained with InstantBlue (Expedeon) (Figure S2). The identity of the sample was confirmed by electrospray injection mass spectrometry (ESI-MS; PNAC facility, Department of Biochemistry, University of Cambridge; Table S3 and Figure S3).

#### *mACPb*

The amino acid sequence for mACPb coded in module 7 of the *MLSB* gene from *Mycobacterium ulcerans* (Uniprot: Q32YM8; residues 13784-13874; Table S2) is identical to that of the ACP domain (mACP<sub>9</sub>) coded by the *MLSA2* gene (Uniprot: Q6MZA5; residues 2050-2140) studied previously by Vance and coworkers [Vance et al., 2016]. As described in that paper, a pET28α vector was used to express N-terminally His<sub>6</sub>-tagged mACPb in *E. coli* Tuner (DE3) cells (Merck). The cells were grown at 37 °C in 1 L of LB medium, prepared according to standard protocols [Sambrook & Russell, 2001], with 30 µg/mL kanamycin (Sigma) for selection, to a 600 nm optical density of 0.8, followed by induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) and incubation at 15 °C for 16 h. The cells were harvested and mACPb was purified and concentrated as described above for mACPa, except His<sub>6</sub>-tag cleavage was performed using restriction grade thrombin (EMD Millipore). All expression and purification steps were monitored by SDS-PAGE (NuPAGE) 4-12 % Bis-Tris gels (Life Technologies) stained with InstantBlue (Expedeon) (Figure S2) and the identity of the sample was confirmed by ESI-MS (PNAC facility, Department of Biochemistry, University of Cambridge; Table S3 and Figure S3).

#### **4. Validation of KR domain secondary structure and reductase activity**

Despite extensive trials, purified samples of mKR<sub>a</sub> and mKR<sub>b</sub> proved to be unsuitable for analysis by mass spectrometry using the Waters Q-TOF micro system in the Protein and Nucleic Acid (PNAC) Facility, Department of Biochemistry, University of Cambridge, due to aggregation during the injection step. To validate these samples, we confirmed that the enzyme domains possessed secondary

structure using circular dichroism (CD) spectroscopy and displayed NADPH-dependent reductase activity against *trans*-1-decalone using a previously established assay [Siskos et al., 2005].

For CD spectroscopy, protein samples were prepared at 0.2 mg/mL in phosphate buffer (45 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaF, 0.01 % (v/v) NaN<sub>3</sub>, pH 7.5). Molar ellipticity data,  $[\theta]$ , was collected at 30 °C using an Aviv model 410 circular dichroism spectrometer between 200 and 260 nm. Runs acquired in triplicate were used to calculate the average spectra displayed in Figure S5, which indicate that both mKR<sub>a</sub> and mKR<sub>b</sub> contain a significant proportion of  $\alpha$ -helical secondary structure. For comparison, the X-ray structure of the KR domain from module 1 of the erythromycin polyketide synthase (PDB code, 2FR0) possesses an  $\alpha$ -helix content of 37% [Keatinge-Clay & Stroud, 2006].

KR domain activities were assessed using a previously designed assay by incubation at 30 °C with NADPH and the substrate mimic *trans*-1-decalone (TDL) in HEPES buffer (25 mM HEPES, 50 mM NaCl, pH 7.5) at 30 °C. Assay reactions were set up in a Costar 96 well UV-transparent plate (Corning) and the progress of the reaction was followed for 20 min by monitoring the depletion of NADPH at 340 nm at 20 s intervals using a Spectramax Plus microplate reader running Softmax Pro (Molecular Devices) with 2 s shaking in between reads. The final assay volume was 110  $\mu$ L, yielding NADPH and KR domain concentrations of 1.3 mM and 8  $\mu$ M, respectively. The KR domain was incubated with NADPH for 20 min prior to addition of TDL. TDL was dissolved in 3 % (v/v) DMSO and its initial well concentration was varied from 0 to 50 mM, while the total amount of DMSO in the well was kept constant. Assays were performed in triplicate for each run to generate plots of the initial rate of consumption of NADPH ( $v_0$ ) against TDL concentration. Fitting to the Michaelis-Menten equation,  $v_0 = V_{\max}[\text{TDL}]/(K_M + [\text{TDL}])$ , using SoftmaxPro v6 yielded values for  $V_{\max}$ ,  $k_{\text{cat}}$  and  $K_M$ , as displayed in Table S4. These results confirm that the KR domain constructs prepared for this work possess kinetic parameters very similar to those obtained in previous studies [Bali & Weissman, 2006].

Overall, since both mKR<sub>a</sub> and mKR<sub>b</sub> contain appropriate secondary structure and show activities similar to those of previously studied equivalents, we concluded that our samples are likely to possess native tertiary structure.

## 5. Preparation of *holo* and acyl-loaded ACP samples

ACP constructs were prepared in the P<sub>ant</sub>-attached *holo* form *in vivo* by co-expression. Two expression vectors, one coding for the ACP construct (pVH-mACPa or pET28 $\alpha$ -mACPb) and the other coding for the broad specificity phosphopantetheinyl transferase Sfp (pET-Sfp) [Quadri et al., 1998], were co-transformed into *E. coli* Tuner(DE3) cells. Procedures for the expression and purification of the *holo* ACP species were as described for the *apo* species in Section 3 above. The modification state

of the ACP domain was confirmed by ESI-MS (PNAC facility, Department of Biochemistry, University of Cambridge; Table S3 and Figure S3).

Loading reactions for acyl-ACP species were set up *in vitro*. An *apo* ACP sample (0.1 mM) was incubated at 27 °C for 2 h with Sfp (4.4 mM) and either acetoacetyl-CoA (2 mM) or  $\beta$ -hydroxybutyryl-CoA (2 mM) in phosphate buffer (45 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 0.01 % (v/v) NaN<sub>3</sub>, pH 7.5) supplemented with 10 mM MgCl<sub>2</sub>. To separate the loaded protein from Sfp and any excess substrate, the mixture was purified by size exclusion chromatography, as described in Section 3 above. The identity of the eluted protein was confirmed by ESI-MS (PNAC facility, Department of Biochemistry, University of Cambridge; Table S3 and Figure S4). For acyl-ACP samples, ESI-MS analysis was carried out after conducting the ITC experiments described in Section 6 below. Although the samples were stored at 4 °C during this 24 h period, both of the acetoacetyl-ACP species proved to be mildly unstable: their mass spectra in panels A and B of Figure S3 show minor species that result from hydrolysis to the *holo* ACP form. However, the absence of signals from the starting material (*apo* ACP species) indicate that in both cases the loading reaction had run to completion. By contrast, no hydrolysis was observed for the two  $\beta$ -hydroxybutyryl-ACP species (panels C and D of Figure S3).

## 6. Isothermal titration calorimetry experiments

ITC measurements of affinity ( $K_D$ ), stoichiometry ( $n$ ) and apparent enthalpy change ( $\Delta H^\circ$ ) were obtained using a VP-ITC microcalorimeter (MicroCal Inc.). Samples for the cell and injectant solutions were prepared in the same buffer (25 mM HEPES, 50 mM NaCl, 0.01 % (v/v) NaN<sub>3</sub>, pH 7.5). Small compounds of interest were dissolved in the flow through from the 5 kDa MWCO Sartorius centrifugal concentrators used to prepare each KR domain sample, to ensure that the buffers were closely matched. All samples were degassed under vacuum using a ThermoVac accessory (MicroCal Inc.) and loaded into the cell and the syringe. The same protocol was used for all experiments, which were conducted at 30 °C with a stirring speed of 300 rpm and a sequence of 29 injections of 10  $\mu$ L, each lasting 7.1 s, and with a 240 s interval between each injection. The first injection was set to a smaller volume (2  $\mu$ L in 3.3 s) to allow the cell and needle solutions to mix, and the resulting heat change was disregarded in the later analysis. Control experiments were run to confirm that dilution heat changes caused by the titration of injectant into the cell solution were negligible. Dilution heat change thermograms for each cell sample were subtracted from the final trace before integration with respect to time to generate an isotherm trace. All isotherm traces were analysed using ORIGIN, version 7.0 (MicroCal, Inc.) with a model that assumed a single binding site. The resulting  $K_D$  values were averaged across 3 technical replicates and a standard deviation was calculated. Representative ITC

thermograms and isotherm plots for experiments with KR samples in the cell and either ACP species or Ppant fragments in the syringe are displayed in Figures S6, S7, S8 and S9.

Interestingly, the heat changes observed on titration of either *apo* mACPa or *apo* mACPb against their cognate KR domains in the absence of NADPH or NADP<sup>+</sup> were miniscule (data not shown); at  $\pm 0.1 \mu\text{cal s}^{-1}$  per injection, these responses were little different from those observed in dilution control experiments (see Fig. 2 of the main text). The lack of a strong heat change signature in the absence of cofactor indicates that binding is weak or non-existent, or that the interaction between the injectant and its binding partner must be isothermic under the conditions of the experiment. Furthermore, in the absence of cofactor titration of *holo* or acyl-ACP species, or of any of the prosthetic group fragments detailed in Scheme 2 of the main text, against either of the KR domains again produced thermograms that resembled dilution control experiments (data not shown). Finally, titration of NADPH or NADP<sup>+</sup> against either of the KR domains yielded similarly weak heat changes (data not shown). Since there is no doubt that the cofactor must bind to the enzyme so that ketoreduction can occur, we conclude that these interactions must be isothermic under the experimental conditions employed here.

**Table S1: Oligonucleotide primers**

PCR primers used for cloning the mycolactone polyketide synthase KR and ACP constructs.

Target	Direction	DNA sequence (5' to 3')
mKR <sub>a</sub>	F	AGCAGCGGCGAAAACCTGTATTTTCAGGGCCGCGCGGAGCGCA
	R	GCCAACTCAGCTTCCTTTTCGGGCTTTGTTAGCGGTAAGCCATTACCTCTGTG
mKR <sub>b</sub>	F	AGCAGCGGCGAAAACCTGTATTTTCAGGGCGACTCATTGATCACCCGCCCC
	R	ACCACACCACGGCGCCGGTAACAAAGCCCGAAAGGAAGCTGAGTTGGC
mACP <sub>a</sub>	F	AGCAGCGGCGAAAACCTGTATTTTCAGGGCTCGACGGCGACGCTCC
	R	GCCAACTCAGCTTCCTTTTCGGGCTTTGTTAACCAGATGCCAGGGATCTGTTC
mACP <sub>b</sub>	F	AGCAGCGGCGAAAACCTGTATTTTCAGGCGCCTCAACGGACTTAGC
	R	GCCAACTCAGCTTCCTTTTCGGGCTTTGTTAATGGCTTTGGGTGAGTCG

**Table S2: Amino acid sequences of protein constructs**

Amino acid sequences of the mycolactone polyketide synthase KR and ACP constructs.

Construct	Amino acid sequence (N-terminal to C-terminal)				
mKR <sub>a</sub>	GRAERTQSEL	DSWRYQVTWL	SSPATPSSIT	LSGIWLLIVP	SELAKTDPVI
	GCAAALAEAHG	ALVTIITIFE	PDFNRSLMGA	SLKDIGSHIS	GVISFLGIHG
	SEFSDSGAVK	TLNLVQAMGD	VHLDVPLWCL	TQGAVSISAD	DLIRCSSAAL
	VWGLGRVVAL	EHPGSWGGLV	DLPESPDDAA	WERLCALLAQ	PTDEDQFAIR
	PSGVFLRRLI	HAPATTTSKS	STAWAPRGTV	LITGGTGALG	AHVARWLAHK
	YESVDLLITS	RRGMAADGAT	EIVDDLRTAG	ASVTVHACDV	TDRTSVEAAI
	AGKSLDAVFH	LAGRHQPTLL	TELEDESFS	ELAPKVHGAQ	VLSDITSNLT
	LSAFVMFSSV	AGIWGGKSQG	AYAAANAFD	SLAEKRRTLQ	LPATSVAWGL
	WAGGGMGDRP	SASGLNLIGL	KSMSADLAVQ	ALSDAIDRPQ	ATLTVASVNW
	DRFYPTFALA	RPRPFLHEIT	EVMAYR		
mKR <sub>b</sub>	GDSLITRPLT	TATGSAPATT	AAGLLHLSWP	PHPDTTDTD	TDTDALRYQV
	IAEPTQQLPR	YLHDLHTSTD	LHTSTTEADV	VVWPVPVPSN	EELQAHQASD
	TAVSSRIHTL	TRQTLTVQD	WLTHPDTTGT	RLVIVTRHGV	STSAHDVPD
	LAHAADVWGLI	RSAQNEHPGR	FTLLDSTDNT	NSDTLTALT	LPTRENQLAI
	RRDTIHIPRL	TRHSSDGALT	APVVVDPEGT	VLITGGTGTL	GALFAEHLVS
	AHGVRLHLLT	SRRGPQAHGA	TDLQQLTDL	GAHVTITACD	ISDPEALAAL
	VNSVPTQHRL	TAVVHTAAVL	ADTPVTELTG	DQLDQVLAPK	IDAALQLHQL
	TYEHNLSAFI	MFSSMAGMIG	SPGQGNIAAA	NTALDALADY	RHRLGLPATS
	LAWGYWQTHT	GLTAHLTDVD	LARMTRLGLM	PIATSHGLAL	FDAALATGQP
	VSIPAPINTH	TLARHARDNT	LAPILSALIT	TPRRR	
mACP <sub>a</sub>	GSTATLLTSK	LAGLTATEQR	AVTRKLVLDQ	AASVLGYAST	ESLDTHESFK
	DLGFDSLIAL	ELRDHLQTAT	GLNLSSTLIF	DHPTPHAVAE	HLLEQIPGIG
mACP <sub>b</sub>	GSHMRLNGLS	PQQQQQTLAT	LVAAATATVL	GHHTPESISP	ATAFKDLGID
	SLTALELRNT	LTHNTGLDLP	PTLIFDHPTP	HALTQHLHTR	LTQSH



**Table S3: ESI-MS analysis of ACP species**

Predicted and experimental molecular masses for ACP species determined by ESI-MS.

ACP species	Predicted mass / Da	Experimental mass / Da
<i>apo</i> mACPa	10596.1	10598.0
<i>holo</i> mACPa	10937.2	10937.3
acetoacetyl-mACPa	11021.2	11021.3
$\beta$ -hydroxybutyryl-mACPa	11023.2	11023.2
<i>apo</i> mACPb	10224.5	10224.8
<i>holo</i> mACPb	10564.8	10564.8
acetoacetyl-mACPb	10648.8	10649.6
$\beta$ -hydroxybutyryl-mACPb	10650.8	10650.8

**Table S4: Kinetic parameters for KR domain constructs**

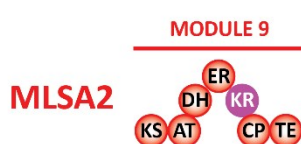
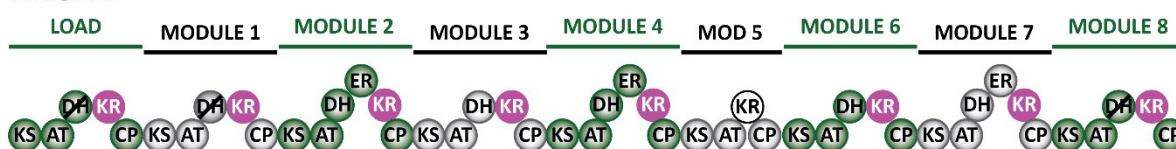
Comparison of kinetic parameters determined for mycolactone KR domain species in the current work with those obtained previously [Bali & Weissman, 2006]. Data shows values for the mean and the standard deviation obtained from three technical replicate experiments.

KR species	$K_M$ / mM	$k_{cat}$ / s <sup>-1</sup>	$k_{cat} / K_M$ / s <sup>-1</sup> M <sup>-1</sup>
mKR <sub>a</sub> (current work)	3.0 (± 0.4)	0.20 (± 0.002)	66 (± 22)
mKR <sub>a</sub> (previous)	3.2 (± 0.5)	0.28 (± 0.01)	88 (± 17)
mKR <sub>b</sub> (current work)	3.1 (± 0.7)	0.40 (± 0.01)	129 (± 14)
mKR <sub>b</sub> (previous)	3.7 (± 0.4)	0.52 (± 0.02)	140 (± 20)

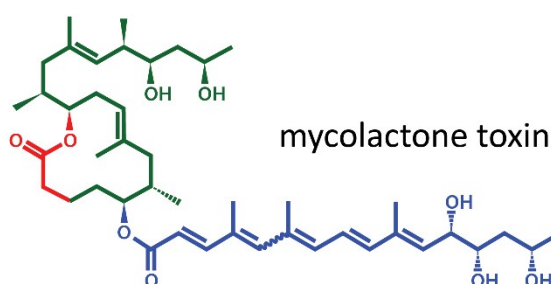
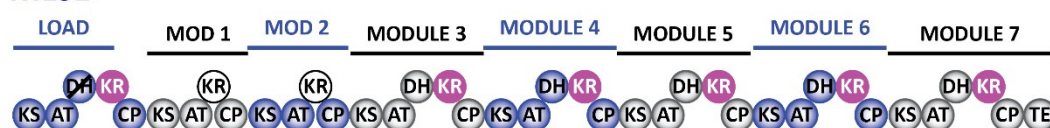
**Figure S1: Module organization for the mycolactone PKS system**

Module organization for the three subunits of the mycolactone PKS system (MLSA1, MLSA2 and MLSB) [Stinear et al., 2004]. The structure of mycolactone is colour coded to match the subunits responsible for synthesizing each segment. A1- and B1-type KR domains are represented in white and magenta, respectively. DH domains that are predicted to be inactive are marked with diagonal black lines. Domain abbreviations: KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; CP, acyl carrier protein; TE, thioesterase.

### MLSA1



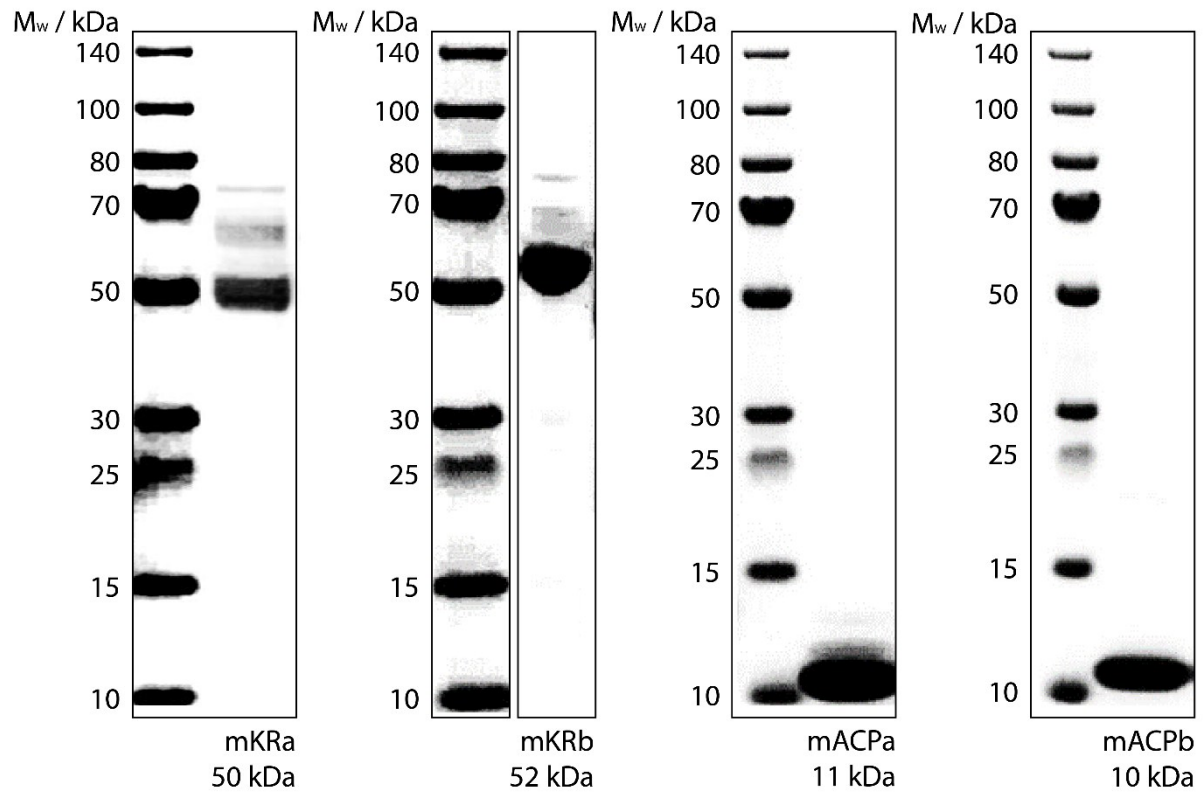
### MLSB



mycolactone toxin

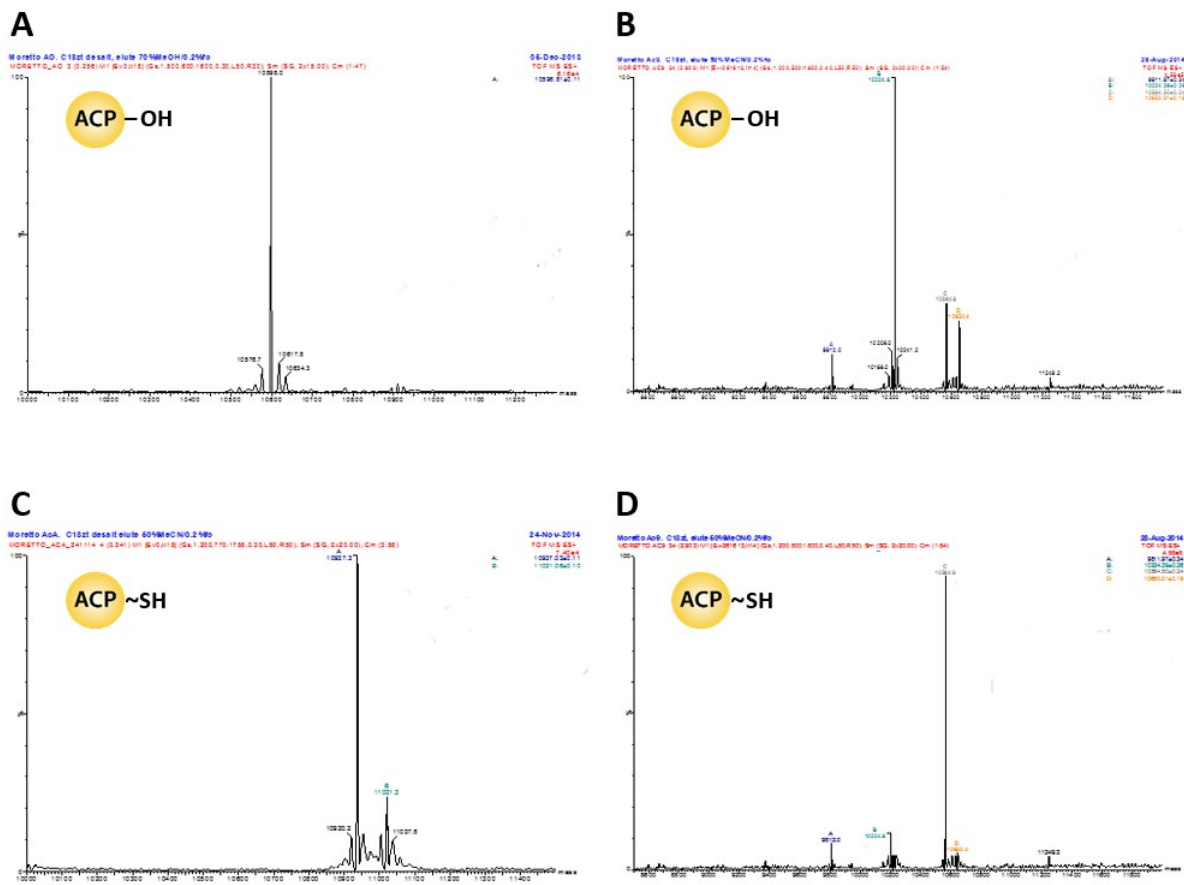
**Figure S2: SDS-PAGE analysis**

Each panel displays a 4-12 % Bis-Tris gel containing Thermo Scientific PageRuler prestrained protein ladder (left lane) and the indicated protein construct after purification by size exclusion chromatography (right lane).

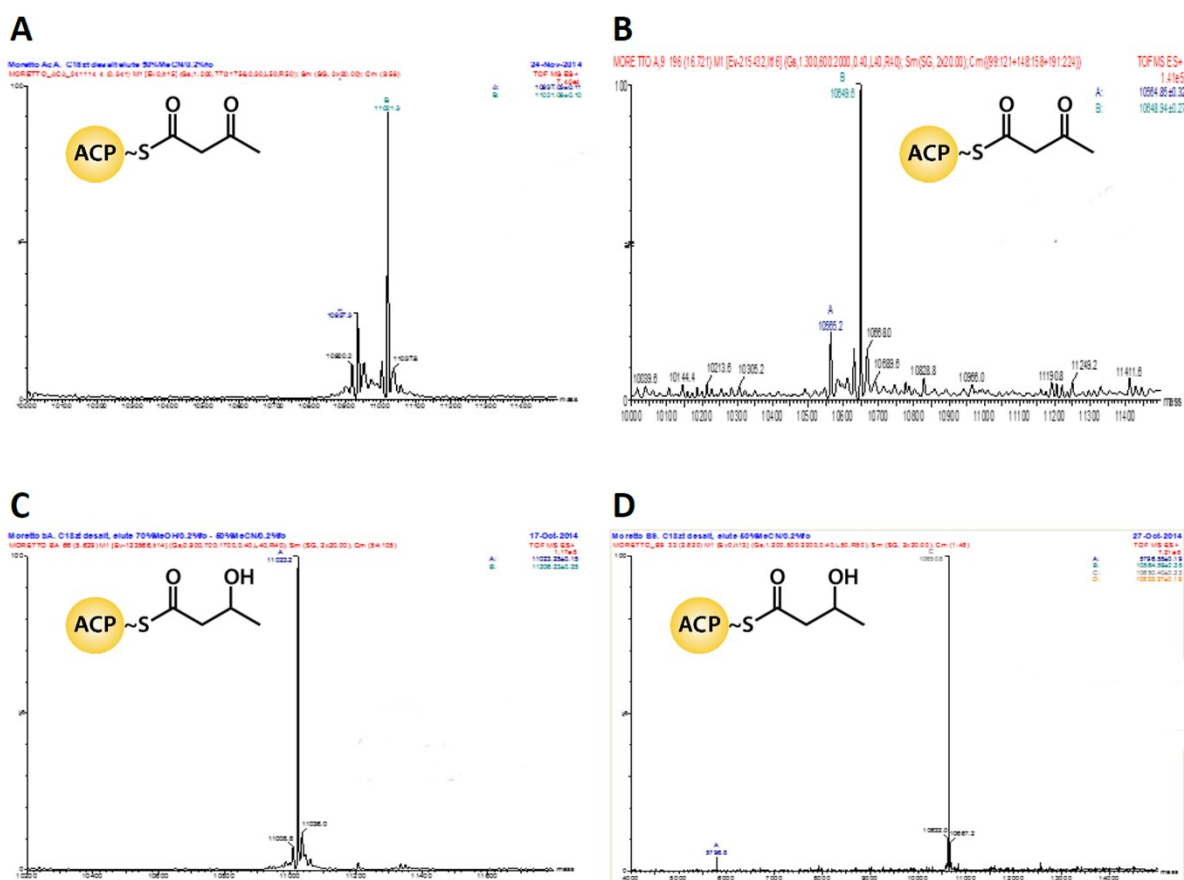


**Figure S3: ESI-MS spectra of *apo* and *holo* ACP species**

Deconvoluted electrospray mass spectra for samples of: (A) *apo* mACP<sub>a</sub>; (B) *apo* mACP<sub>b</sub>; (C) *holo* mACP<sub>a</sub>; and (D) *holo* mACP<sub>b</sub>.

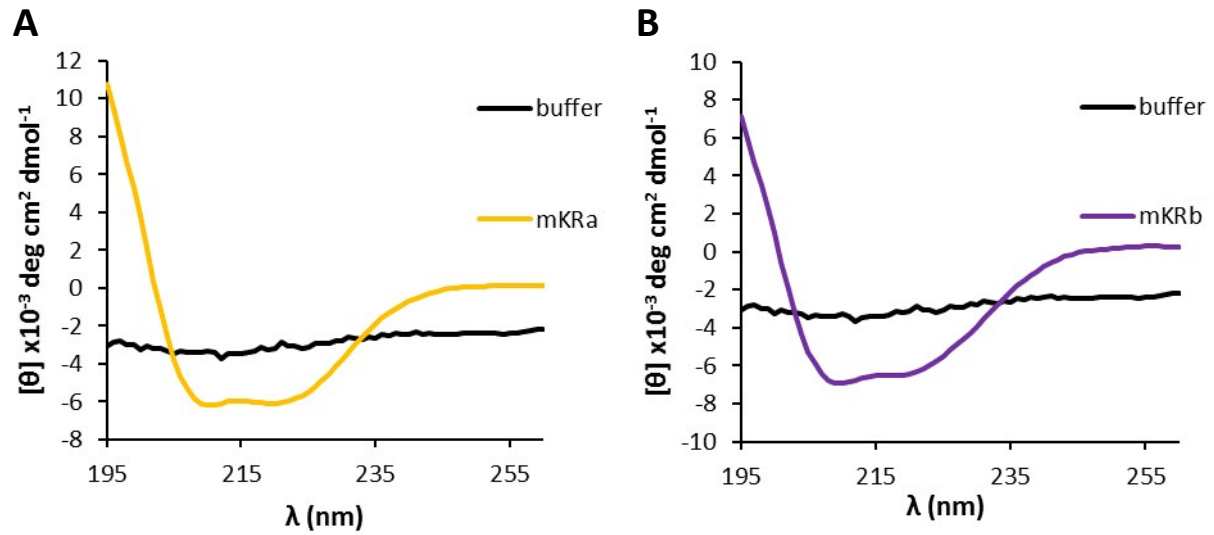


Deconvoluted electrospray mass spectra for samples of: (A) acetoacetyl-mACPa; (B) acetoacetyl-mACPb; (C)  $\beta$ -hydroxybutyryl-mACPa; and (D)  $\beta$ -hydroxybutyryl-mACPb.



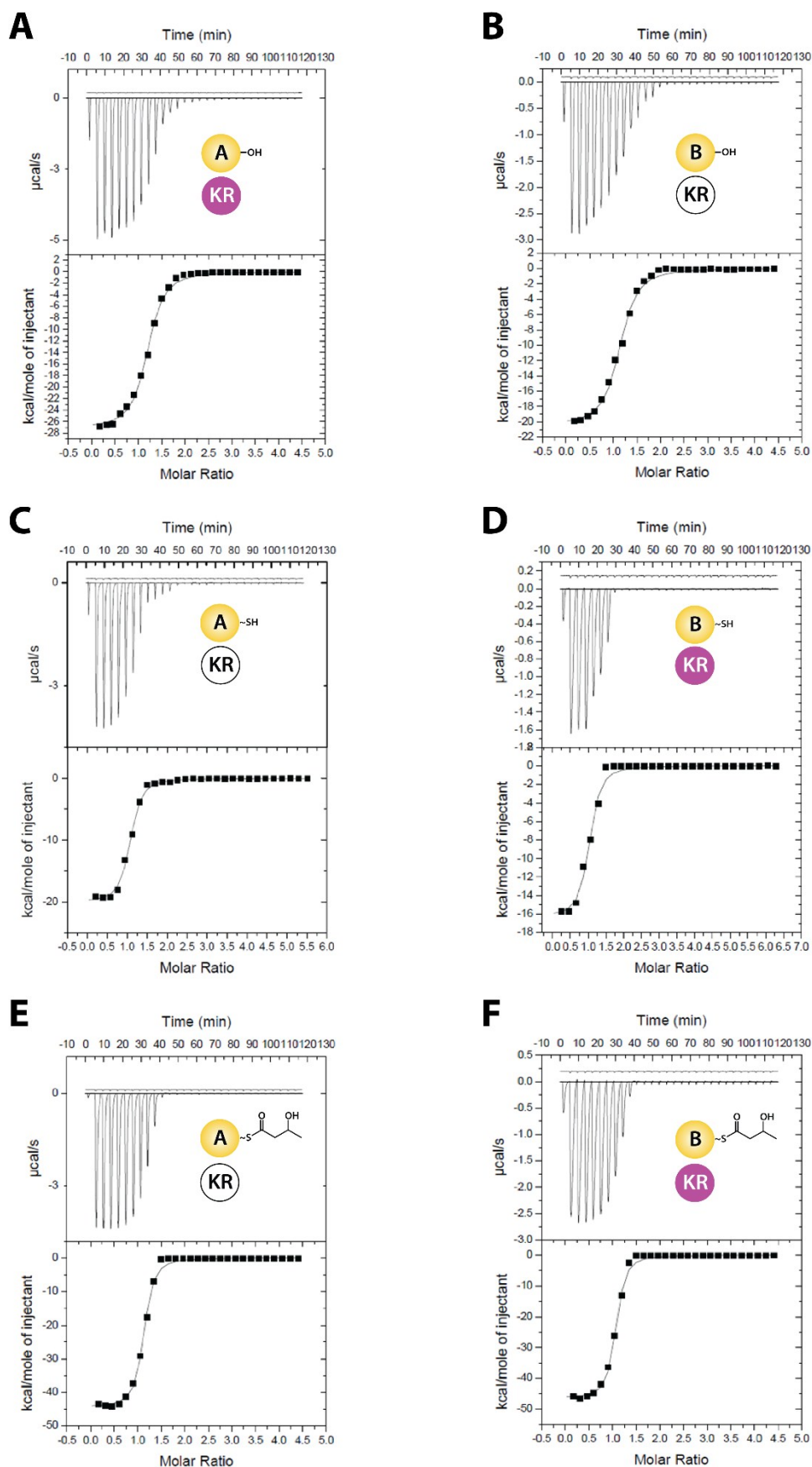
**Figure S5: Circular dichroism spectra of KR domain constructs**

CD spectra are displayed for samples of (A) mKRa and (B) mKRb, both showing minima at 209 nm and 222 nm, which are characteristic  $\alpha$ -helical secondary structure.



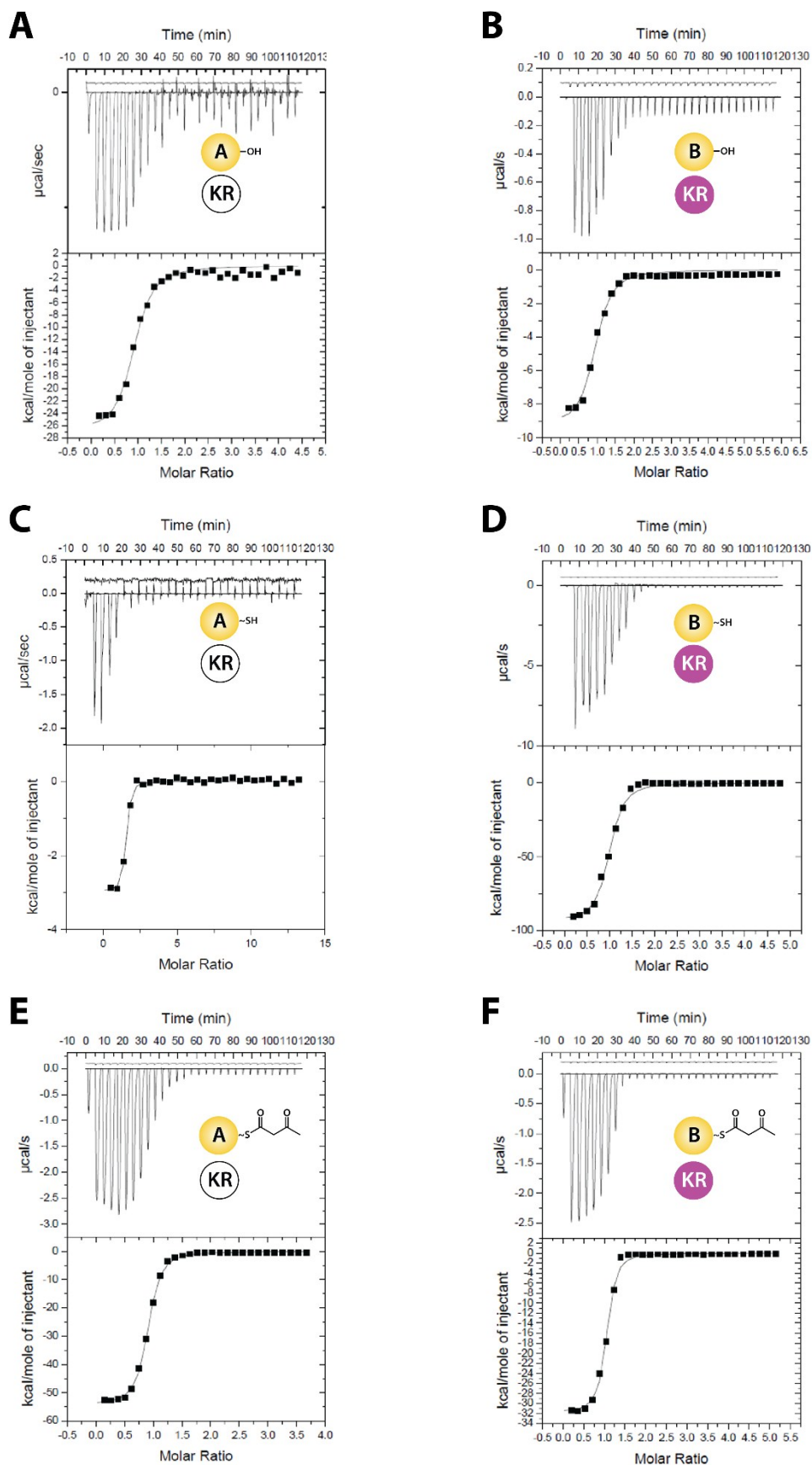
**Figure S6: Isothermal titration calorimetry data I**

Representative ITC thermograms (upper panels) and isotherm plots (lower panels), all in the presence of excess NADPH, showing consecutive injections of of: (A) *apo* mACPa against mKRb; (B) *apo* mACPb against mKRb; (C) *holo* mACPa against mKRb; (D) *holo* mACPb against mKRb; (E)  $\beta$ -hydroxybutyryl-mACPa against mKRb; and (F)  $\beta$ -hydroxybutyryl-mACPb against mKRb. Thermogram traces for dilution control experiments are shown at the top of each upper panel.



### Figure S7: Isothermal titration calorimetry data II

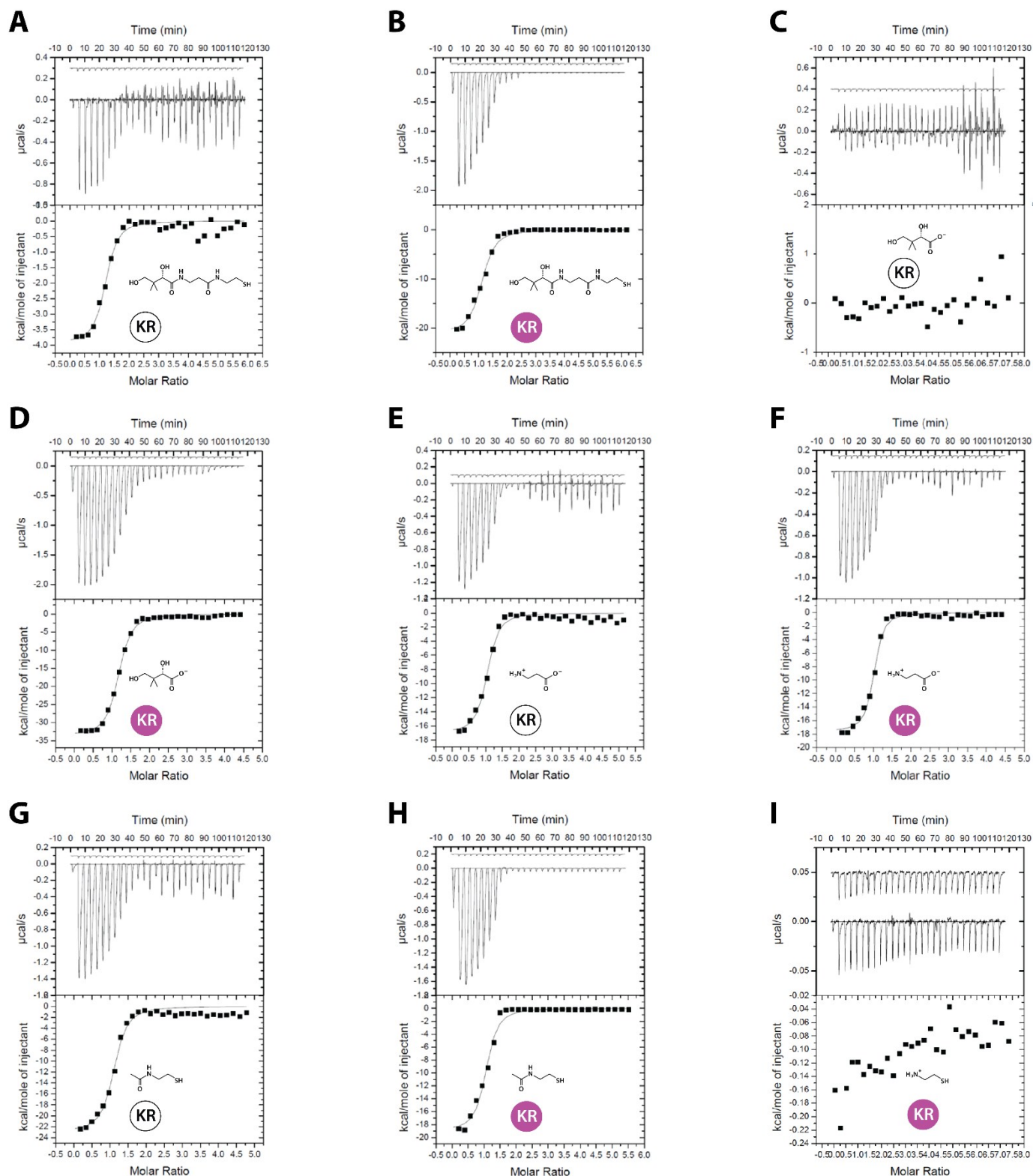
Representative ITC thermograms (upper panels) and isotherm plots (lower panels), all in the presence of excess NADP<sup>+</sup>, showing consecutive injections of: (A) *apo* mACPa against mKR<sub>a</sub>; (B) *apo* mACPb against mKR<sub>b</sub>; (C) *holo* mACPa against mKR<sub>a</sub>; (D) *holo* mACPb against mKR<sub>b</sub>; (E) acetoacetyl-mACPa against mKR<sub>a</sub>; and (F) acetoacetyl-mACPb against mKR<sub>b</sub>. Thermogram traces for dilution control experiments are shown at the top of each upper panel.





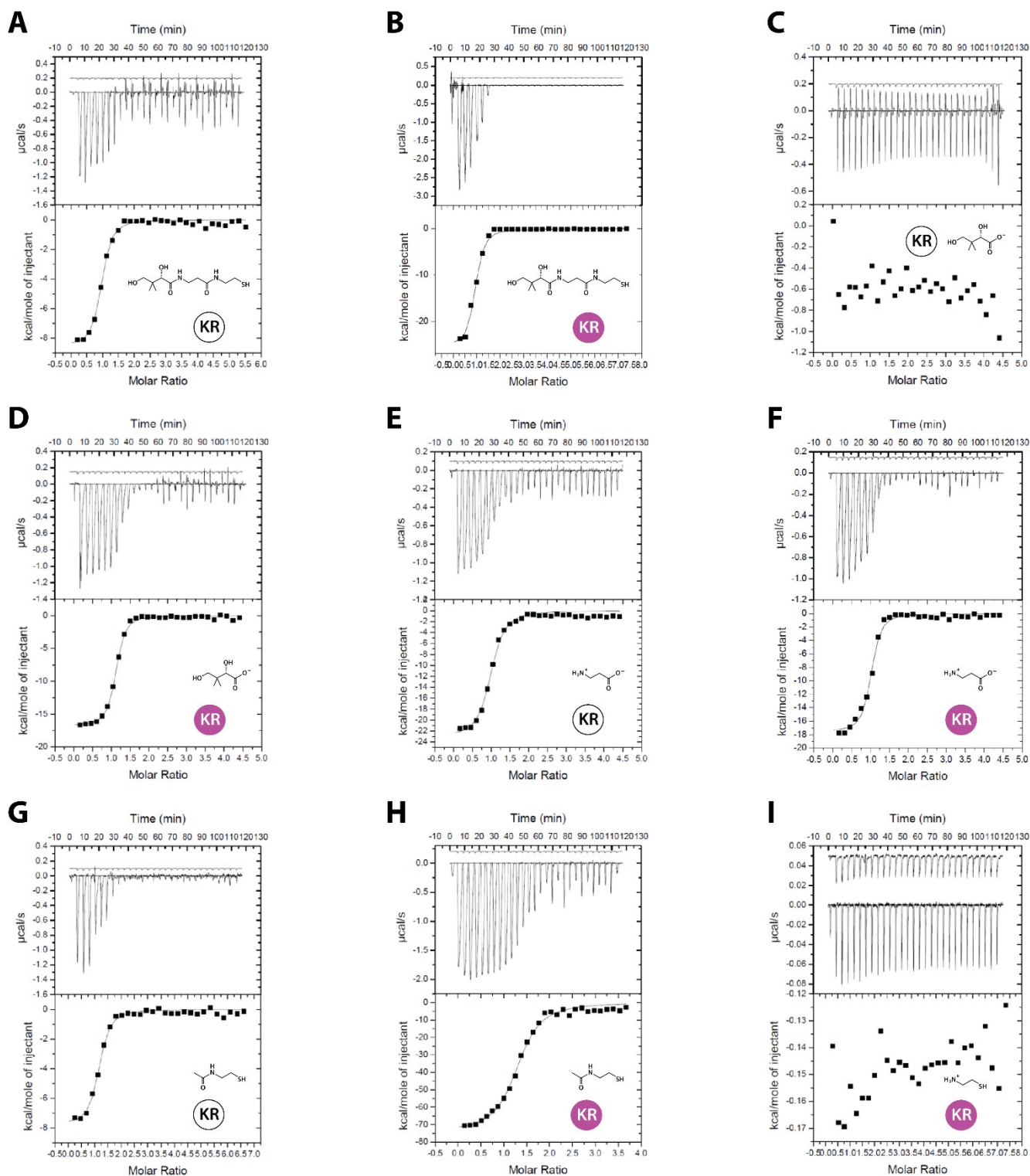
**Figure S8: Isothermal titration calorimetry data III**

Representative ITC thermograms (upper panels) and isotherm plots (lower panels), all in the presence of excess NADPH, showing consecutive injections of: (A) R-pantetheine against mKR<sub>a</sub>; (B) R-pantetheine against mKR<sub>b</sub>; (C) pantoate against mKR<sub>a</sub>; (D) pantoate against mKR<sub>b</sub>; (E)  $\beta$ -alanine against mKR<sub>a</sub>; (F)  $\beta$ -alanine against mKR<sub>b</sub>; (G) *N*-acetyl cysteamine against mKR<sub>a</sub>; (H) *N*-acetyl cysteamine against mKR<sub>b</sub>; and (I) cysteamine against mKR<sub>b</sub>. Thermogram traces for dilution control experiments are shown at the top of each upper panel.



**Figure S9: Isothermal titration calorimetry data IV**

Representative ITC thermograms (upper panels) and isotherm plots (lower panels), all in the presence of excess NADP<sup>+</sup>, showing consecutive injections of: (A) R-pantetheine against mKR<sub>a</sub>; (B) R-pantetheine against mKR<sub>b</sub>; (C) pantoate against mKR<sub>a</sub>; (D) pantoate against mKR<sub>b</sub>; (E) β-alanine against mKR<sub>a</sub>; (F) β-alanine against mKR<sub>b</sub>; (G) *N*-acetyl cysteamine against mKR<sub>a</sub>; (H) *N*-acetyl cysteamine against mKR<sub>b</sub>; and (I) cysteamine against mKR<sub>b</sub>. Thermogram traces for dilution control experiments are shown at the top of each upper panel.



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