Supporting Information

Catalytic promiscuity of ancestral esterases and hydroxynitrile lyases

Titu Devamani,[a] Alissa M. Rauwerdink,[a] Mark Lunzer,[b] Bryan J. Jones,[a] Joanna L. Mooney,[a] Maxilmilien Alaric O. Tan,[c] Zhi-Jun Zhang,[d] Jian-He Xu,[d] Antony M. Dean[b,e]*, Romas J. Kazlauskas[a]*

[a]*University of Minnesota, Department of Biochemistry, Molecular Biology & Biophysics and The Biotechnology Institute, 1479 Gortner Avenue, Saint Paul, MN 55108 USA*

[b]*University of Minnesota, Department of Ecology, Evolution & Behavior and The Biotechnology Institute, 1479 Gortner Avenue, Saint Paul, MN 55108 USA*

[c]*University of Minnesota, Department of Chemistry, 220 Pleasant Street, Minneapolis, MN 55455 USA*

[d]*East China University of Science and Technology, School of Biotechnology, Meilong Road 130, Shanghai 200237 P. R. China*

[e]*Sun Yat-sen University, Institute of Ecology and Evolution, No.135, Xinggang West Road, Guangzhou, 510275 P. R. China*

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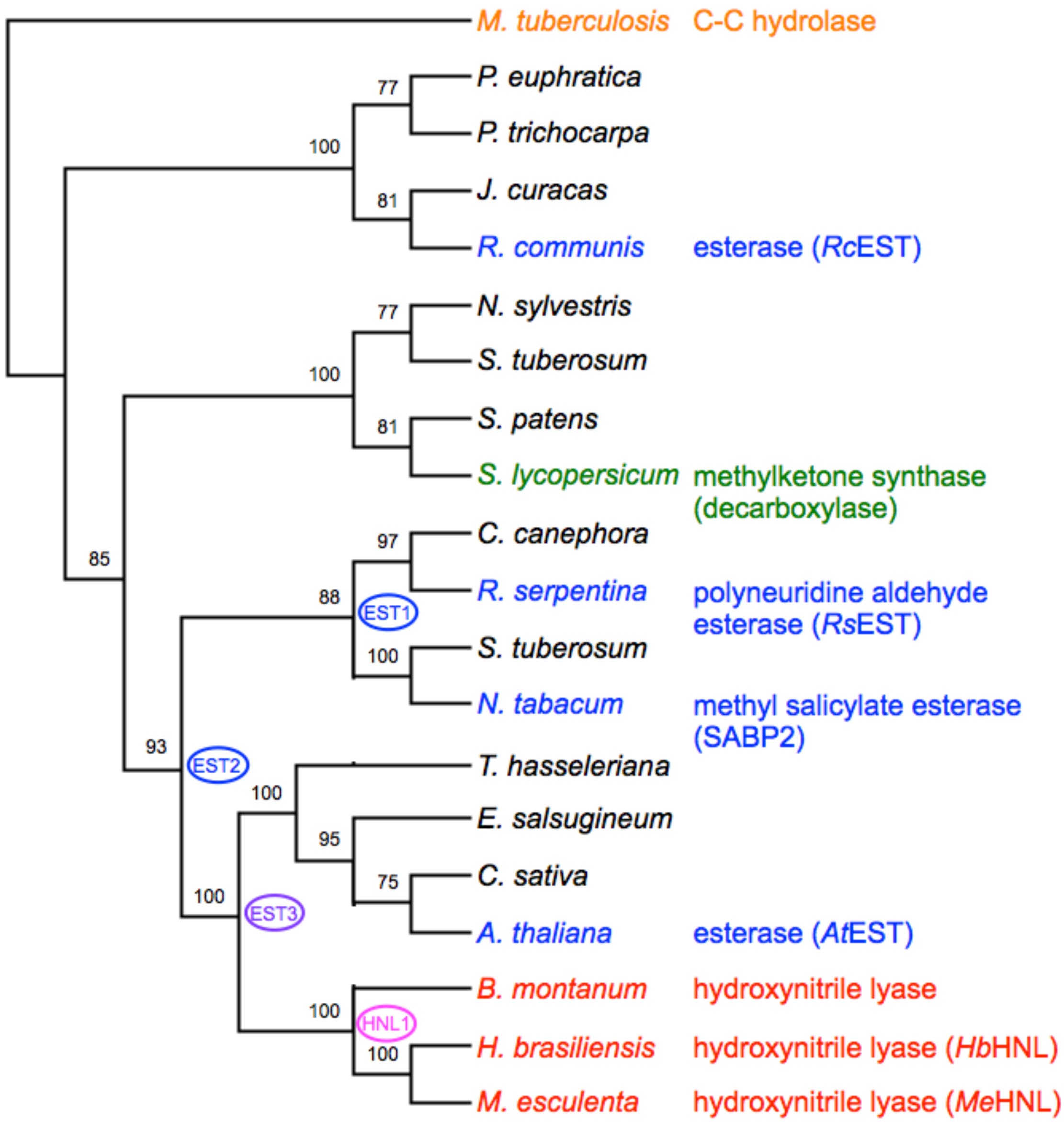

Figure S1. Cladogram showing the hydroxynitrile lyases (red text) in the α/β-hydrolase fold family cluster within a larger group of plant esterases (blue text). The decarboxylase (green text) is more distantly related as are meta-cleavage product hydrolases (orange text, connected by dotted lines). Black text indicates presumed esterases with unverified functions. Ancestral enzymes were reconstructed at the labelled nodes. The numbers on the lines are bootstrap values (in percent). Values above 70% indicate that tree is draw correctly.[1]

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RcEST     MGNRFICMTKKDAGDNQGSR SKRLGRSQRKLLADEDLLHR QALSMALHQHQLSQRFEGSM SRRIGSTTSRKRNLSDPFSN GKQVPDFAENIKFKKFILVH
MKS1      -------------------- -------------------- -------------------- -------------------- ---MEKSMSPFVKKHFVLVH
RsEST     -------------------- -------------------- -------------------- -------------------- ---MHSAANAKQQKHFVLVH
SABP2     -------------------- -------------------- -------------------- -------------------- ---------MKEGKHFVLVH
AtEST     -------------------- -------------------- -------------------- -------------------- ---------MERKHHFVLVH
MeHNL     -------------------- -------------------- -------------------- -------------------- ----------MVTAHFVLIH
HbHNL     -------------------- -------------------- -------------------- -------------------- ----------MAFAHFVLIH
HNL1-ML   -------------------- -------------------- -------------------- -------------------- ----------MAWAHFVLIH
HNL1-NJ   -------------------- -------------------- -------------------- -------------------- ----------MAVAHFVLIH
HNL1      -------------------- -------------------- -------------------- -------------------- ----------MATAHFVLIH
EST3-ML   -------------------- -------------------- -------------------- -------------------- ---------MKRKQHFVLVH
EST3-NJ   -------------------- -------------------- -------------------- -------------------- ---------MERKKHFVLVH
EST3      -------------------- -------------------- -------------------- -------------------- ---------MQAKAHFVLVH
EST1      -------------------- -------------------- -------------------- -------------------- -----MAEMKQQTKHFVLVH
EST2      -------------------- -------------------- -------------------- -------------------- -----MAEMKNRTKHFVLVH

RcEST     GEGFGAWCWYKTVALLEEAG LLPTALDLTGSGIHLTDTNS VTKLADYSQPLINYLENLPE DEKVILVGHSTGGACISLAL EHFPQKISKAIFLCATMVSD
MKS1      TAFHGAWCWYKIVALMRSSG HNVTALDLGASGINPKQALQ IPNFSDYLSPLMEFMASLPA NEKIILVGHALGGLAISKAM ETFPEKISVAVFLSGLMPGP
RsEST     GGCLGAWIWYKLKPLLESAG HKVTAVDLSAAGINPRRLDE IHTFRDYSEPLMEVMASIPP DEKVVLLGHSFGGMSLGLAM ETYPEKISVAVFMSAMMPDP
SABP2     GACHGGWSWYKLKPLLEAAG HKVTALDLAASGTDLRKIEE LRTLYDYTLPLMELMESLSA DEKVILVGHSLGGMNLGLAM EKYPQKIYAAVFLAAFMPDS
AtEST     NAYHGAWIWYKLKPLLESAG HRVTAVELAASGIDPRPIQA VETVDEYSKPLIETLKSLPE NEEVILVGFSFGGINIALAA DIFPAKIKVLVFLNAFLPDT
MeHNL     TICHGAWIWHKLKPALERAG HKVTALDMAASGIDPRQIEQ INSFDEYSEPLLTFLEKLPQ GEKVIIVGESCAGLNIAIAA DRYVDKIAAGVFHNSLLPDT
HbHNL     TICHGAWIWHKLKPLLEALG HKVTALDLAASGVDPRQIEE IGSFDEYSEPLLTFLEALPP GEKVILVGESCGGLNIAIAA DKYCEKIAAAVFHNSVLPDT
HNL1-ML   TICHGAWIWYKLKPLLEAAG HKVTALDLAASGIDPRQIEQ IGSFDEYSEPLLTFMESLPQ GEKVILVGESCGGINIAIAA DKYPEKIAAAVFHNALMPDT
HNL1-NJ   TICHGAWIWYKLKPLLESAG HKVTALDLAASGIDPRQIEQ VGTFEEYSEPLLTFLESLPE GEKVILVGESCGGINIALAA DKYPEKISAAVFHNALMPDT
HNL1      TICHGAWIWYKLKPLLEAAG HKVTALDLAASGIDPRQIEQ INTFDEYSEPLLTFMESLPQ GEKVILVGESCGGLNIALAA DKYPEKISAAVFHNALMPDT
EST3-ML   NACHGAWIWYKLKPLLEAAG HRVTALDLAASGIDPRQIEE VETFDEYSEPLMEFMESLPE NEKVILVGHSFGGINIALAA DKFPEKISVAVFLNAFMPDT
EST3-NJ   GACHGAWIWYKLKPLLESAG HRVTALDLAASGIDPRQIEA VGTFEEYSEPLLEFLASLPE NEKVILVGHSFGGINIALAA DKFPEKISVAVFVNAFMPDT
EST3      NICHGAWIWYKLKPLLEAAG HKVTAIDLAASGIDPRQIEQ VNTFDEYSEPLLEFMESLPQ NEKVILVGHSFGGLNIALAA DKFPEKISVAVFLNALMPDT
EST1      GACHGAWIWYKLKPLLEAAG HRVTALDLAASGINPRKIEE VHTFDEYSEPLMELMASLPP NEKVILVGHSFGGLNLALAM EKFPEKISVAVFLTAFMPDT
EST2      GACHGAWVWYKLKPLLEAAG HRVTALDLAASGINPKKIEE VHTFDEYSEPLMELMASLPP NEKVILVGHSLGGLNLALAM EKFPEKISVAVFLTAFMPDT

RcEST     GQRPFDVFAEELGSA-ERFM QESEFLIYGNGKDKAPTGFM FEKQQMKGLYFNQSTTKDVA LAMVCMRPIPLG---PVMEK LSLSPEKYGTGRRFFIQTLD
MKS1      NIDATTVCTKAGSAVL--GQ LD-NCVTYENGPTNPPTTLI AGPKFLATNVYHLSPIEDLA LATALVRPLYLYLAEDISKE VVLSSKRYGSVKRVFIVATE
RsEST     NHSLTYPFEKYNEKCPADMM LDSQFSTYGNPENP-GMSMI LGPQFMALKMFQNCSVEDLE LAKMLTRPGSLF-FQDLAKA KKFSTERYGSVKRAYIFCNE
SABP2     VHNSSFVLEQYNERTPAENW LDTQFLPYGSPEEP-LTSMF FGPKFLAHKLYQLCSPEDLA LASSLVRPSSLF-MEDLSKA KYFTDERFGSVKRVYIVCTE
AtEST     THVPSHVLDKYMEMPG--GL GDCEFSSHET-RNGTMSLLK MGPKFMKARLYQNCPIEDYE LAKMLHRQGSFF-TEDLSKK EKFSEEGYGSVQRVYVMSSE
MeHNL     VHSPSYTVEKLLESLP--DW RDTEYFTFTNITGETITTMK LGFVLLRENLFTKCTDGEYE LAKMVMRKGSLF-QNVLAQR PKFTEKGYGSIKKVYIWTDQ
HbHNL     EHCPSYVVDKLMEVFP--DW KDTTYFTYTKD-GKEITGLK LGFTLLRENLYTLCGPEEYE LAKMLTRKGSLF-QNILAKR PFFTKEGYGSIKKIYVWTDQ
HNL1-ML   VHNPSYVLDKFMEVFP--DW KDSEFSNYTYG-NDTITALK LGPKLMKENLYTNCPPEDYE LAKMLVRKGSLF-QEDLAKR ENFTKEGYGSIKRIYVYGDE
HNL1-NJ   VHSPSYVLDKMFEVFP--DW KDSVFSNYTNGSNDTITALK LGPKLMKENIYTNCPIEDYE LAKMLVRKGSLF-QEDLAKR EKFTEEGYGSIKRVYVYGDE
HNL1      EHSPSYVVDKFMEVFP--DW KDTEFSTYTSN-NETITGMK LGFKLMRENLYTNCPIEDYE LAKMLTRKGSFF-QNDLAQR PKFTEEGYGSIKRVYVWTDE
EST3-ML   THSPSYVLDKFMEMFP--DW KDSEFSSYES-RNGTMTSLK MGPKFMKNKLYQECPVEDYE LAKMLVRQGSFF-KEDLSKK EKFSEEGYGSVKRVYIMGDE
EST3-NJ   THSPSYVLDKMFERFP--PW LDSEFSPYENPSNNTMTSLK FGPKFMKEKLYQNCPIEDYE LAKMLVRPGSLF-KEDLSKK EKFSEEGYGSVKRVYIVGDE
EST3      EHSPSYVVDKYMEVPP--GW RDTEFSPYGSP-NETMTSMK LGFKLMRANLYQNCPIEDYE LAKMLVRQGSFF-QEDLAKR KKFTEEGYGSVKRVYVMTNE
EST1      EHRPSYVLEKYNERTPAEAW LDTQFSPYGMPEEP-LTSML FGPKFMANKLYQNCPIEDLE LAKMLVRPGSLF-IEDLSKA KKFSDEGYGSVQRVYIVCNE
EST2      EHRPSYVLEKYNERTPAEAW LDTQFSPYGNPEEP-LTSML FGPKFMANKLYQLSPIEDLE LAKMLVRPGSLF-IEDLSKA KKFSDEGYGSVPRVYIVCNE

RcEST     DHALSPDVQEKLVRENPPEG VFKIKGSDHCPFFSKPQSLH KILLEIAQIP---------- -------
MKS1      NDALKKEFLKLMIEKNPPDE VKEIEGSDHVTMMSKPQQLF TTLLSIANKYK--------- -------
RsEST     DKSFPVEFQKWFVESVGADK VKEIKEADHMGMLSQPREVC KCLLDISDS----------- -------
SABP2     DKGIPEEFQRWQIDNIGVTE AIEIKGADHMAMLCEPQKLC ASLLEIAHKYN--------- -------
AtEST     DKAIPCDFIRWMIDNFNVSK VYEIDGGDHMVMLSKPQKLF DSLSAIATDYM--------- -------
MeHNL     DKVFLPDFQRWQIANYKPDK AYQVQGGDHKLQLTKTEEVA HILQEVADAYA--------- -------
HbHNL     DEIFLPEFQLWQIENYKPDK VYKVEGGDHKLQLTKTKEIA EILQEVADTYN--------- -------
HNL1-ML   DKIFTEEFQRWQIDNYKPDK VYVVPGGDHKLMLSKVNELF QILQEVADTYANLLAVGGGH HHHHH*-
HNL1-NJ   DKIFLEEFQRWQINNYKPDK VYEVPGGDHKLMLSKVNELF QILQEVADTYASLLAVAGGG HHHHHH*
HNL1      DKIFPPEFQLWQIENYKPDK VYRVQGGDHKLQLSKTNELA EILQEVADTYADLLAVAGGG HHHHHH-
EST3-ML   DKVIPEEFQRWMIDNFNVNK VYEIQGGDHMLMLSKPQELF DSLQEIADNYAGGGHHHHHH *------
EST3-NJ   DKAIPEEFQRWMIDNFPVDK VYEIDGGDHMLMLSKPQELF DCLQEIADKYASLTSVAGGG HHHHHH*
EST3      DKAFPPEFQLWQIENYNPNK VYEVKGGDHKVQLSKTQELA DILQEVADNYADLLDVLGGG HHHHHH-
EST1      DKAIPEEFQRWMIENSGVNK VMEIKGADHMPMFSKPQELC QCLLEIANKYAKAGDPLGGG HHHHHHH
EST2      DKAIPEEFQRWMIENSGVNE VMEIKGADHMPMFSKPQELC QCLLEIANKYAKAGDPLGGG HHHHHH-
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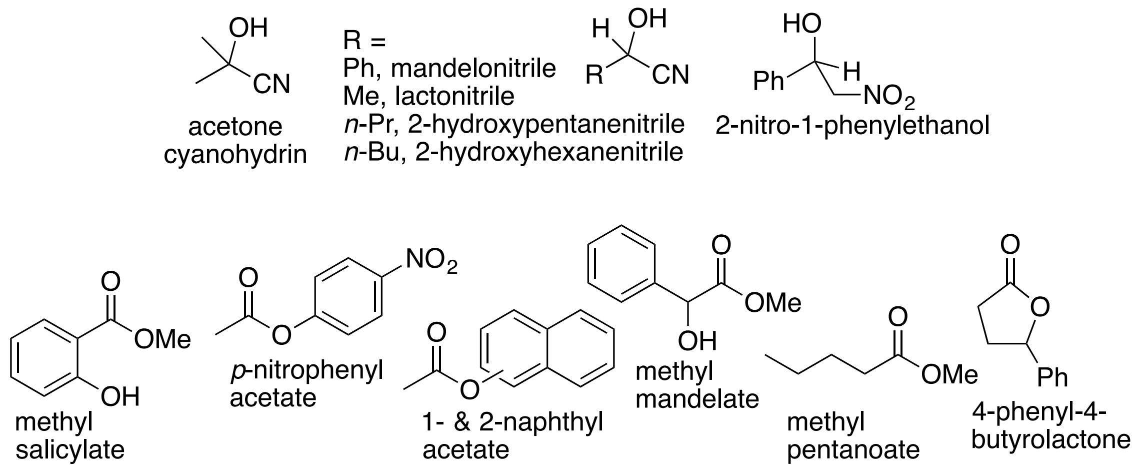
Figure S2. Alignment of the amino acid sequences of selected modern α/β-hydrolases and reconstructed ancestral enzymes. Yellow highlights the conserved catalytic triad, while blue highlights the additional residues needed to interconvert hydroxynitrile lyase and esterase activity.[2, 3] Alignment made using the Clustal Omega algorithm within SeaView 4.4.0 (http://doua.prabi.fr/software/seaview). Abbreviations: *Rc*EST: Polyneuridine-aldehyde esterase precursor, putative from *Ricinus communis* NCBI Reference Sequence: XP_002510769.1; MKS1: methylketone synthase I from *Lycopersicon hirsutum f. glabratum* GenBank: ADK38535.1; *Rs*EST: Polyneuridine-aldehyde esterase from *Rauvolfia serpentina* UniProtKB/Swiss-Prot: Q9SE93.1; SABP2: Salicylic acid-binding protein 2 from *Nicotiana tabacum* UniProtKB/Swiss-Prot: Q6RYA0.1; AtEST: EST5 from *Arabidopsis thaliana* (shows R-selective cleavage of mandelonitrile) NCBI Reference Sequence: NP_196592.1; MeHNL: S hydroxynitrile lyase from *Manihot esculenta* GenBank: AAV52632.1; *Hb*HNL: S hydroxynitrile lyase from *Hevea brasiliensis* UniProtKB/Swiss-Prot: P52704.1

Table S1. Pairwise amino acid sequence identities of modern and ancestral enzymes.

	SABP 2	*Rs*-EST	*Rc*ESt	EST2	EST1	EST3-NJ	EST3-ML	EST3	HNL1 -NJ	HNL1 -ML	HNL1	AtEST	*Me*HNL
***Hb*HNL**	44	41	21	48	49	56	58	67	79	75	79	47	76
***Me*HNL**	41	39	21	44	45	53	54	63	67	67	74	45	
***At*EST**	50	46	24	58	60	73	78	66	59	57	55		
HNL1	49	48	23	57	59	68	69	84	84	84			
HNL1-ML	51	46	24	56	58	71	71	74	91				
HNL1-NJ	50	49	25	58	59	77	71	75					
EST3	55	52	26	66	69	73	76						
EST3-ML	60	54	27	69	71	85							
EST3-NJ	59	56	29	72	73								
EST1	70	64	29	96									
EST2	71	62	29										
***Rc*Est**	28	27											
***Rs*Est**	56												
SABP2													

[a]Background color emphasizes the amino acid sequence similarities ranging from 29% (red) to 96% (green).

Table S2. Cyanohydrin cleavage and ester hydrolysis catalyzed by modern and ancestral enzymes.[a]



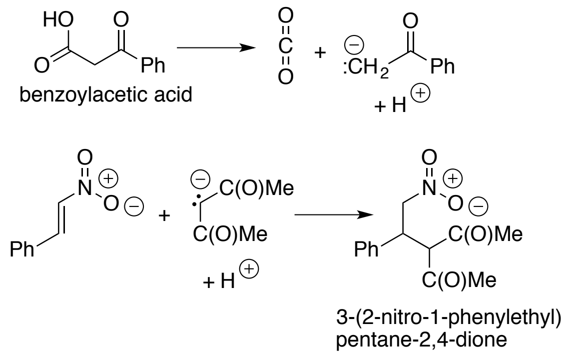
Enzyme	**rate of cyanohydrin cleavage, min^{-1}**						**rate of ester hydrolysis, min^{-1}**					
	acetone cyanohydrin	mandelonitrile	lactonitrile	2-OH pentanenitrile	2-OH hexanenitrile	2-nitro-1-phenylethanol[c]	methyl salicylate	1-naphthyl acetate	2-naphthyl acetate	methyl mandelate	methyl pentanoate	4-phenyl-4-butyrolactone
*Hb*HNL	2400 ± 100[b]	1530 ±130[e b] (>199, *S*)	50 ±11	7.2 ±0.8[b]	24 ±2[b]	7.2[f] ±0.3 *(49, S)*	0.066 ±0.006	<0.008	<0.008	<0.02	<0.44	<0.0001
*Me*HNL	12600 ±800	1340 ±20[b] (>49, S)	13 ±1	0.66±0.06	1.1 ±0.1	0.3 (1.1, *S*)	<0.09	<0.4	<0.4	<0.09	<0.44	<0.0001
HNL1-ML	720 ± 60	170 ± 20 (4, *S*)	7.2 ±0.6	0.14 ±0.01	0.17 ±0.02	3.4 ±0.4 (5.6, *S*)	0.66 ±0.08	0.054 ±0.005	0.13 ±0.01	<0.1	0.45 ±0.04	<0.0001
HNL1-NJ	350 ±10	60 ±5 (32, *S*)	6.4 ±0.7	0.011 ±0.001	0.013 ±0.001	9.6 ±0.6 (32, *S*)	0.030 ±0.004	0.0090 ±0.0001	0.0054 ±0.0007	<0.006	1.0 ± 0.1	0.0007 ±0.0003 (14, *S*)
HNL1	880 ±70[b]	340 ±10[b] (49, *S*)	5.0 ±0.2	0.5 ±0.1[b]	0.42 ±0.09[b]	14 ±1 (32, *S*)	0.048 ±0.006	0.016 ±0.002	0.024 ±0.003	<0.006	<0.44	<0.0001
EST3-ML	0.078 ±0.006	36 ±9 (6.7, *R*)	<1.2	<0.06	<0.06	<0.5	<0.006	0.0036 ±0.0004	0.0084 ±0.0009	<0.006	<0.44	<0.0001

EST3-NJ	<0.06	<0.5	<1.2	<0.06	<0.06	<0.5	0.13 ±0.01	0.28±0.04	0.46 ±0.04	0.11±0.01 (1.0)	46 ± 4	0.00022 ±0.00011 (15, *R*)
EST3	<0.06	<0.5	<1.2	<0.06	<0.06	<0.5	<0.012	<0.06	0.0024 ±0.0002	<0.006	0.66 ±0.05	<0.0001
EST2	<0.06	0.70 ±0.09 (2.1, *S*)	<1.2	<0.06	0.21 ±0.03	<0.5	14 ±1	0.084 ±0.006	1.5±0.1	1.5 ±0.1 (1.3, *S*)	340 ±20	0.00025 ±0.00007 (55, *R*)
EST1	<0.06	6.5 ±0.9 (2.3, *S*)	<1.2	<0.06	0.034±0.006	<0.5	26 ±3	0.096 ±0.008	2.2±0.2	0.45 ±0.05 (1.0)	140 ±10	0.00038 ±0.0001 (38, *R*)
SABP2	<0.06	<0.5	<1.2	<0.06	<0.06	<0.5	0.52 ±0.05[c]	0.012±0.007	0.018±0.002	2.8 ±0.3 (1.0)	198 ±2	0.00077 ±0.0001 (1.0)
*Rc*EST	<0.06	<0.5	<0.18	<0.4	<0.4	<0.5	7.5 ±0.6	0.12±0.01	0.18±0.02	0.013 ±0.001 (1.0)	2.8 ±0.2	<0.0001
*Rs*EST	<0.06	<0.5	<0.18	<0.1	<0.1	<0.5	0.042 ±0.004	0.042 ±0.003	0.012 ±0.002	0.12 ±0.01 (1.2, *R*)	7.1±0.6	0.00066±0.00007 (13, *S*)
*At*EST	<0.06	2530 ±70[b] (>199, *R*)	<1.2	<0.06	<0.06	4.6 ±3 (13, *R*)	<0.02	1.7 ±0.2	0.038±0.004	<0.006	0.72 ±0.06	0.00054 ±0.0003 (8.3, *R*)

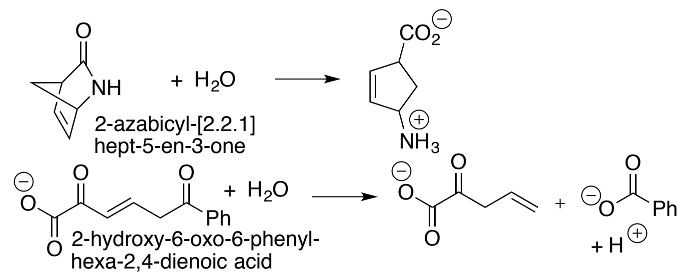
[a] Error limits are standard deviations from three measurements. Rates (min^{-1}) correspond to hydrolysis or cleavage of the substrate shown determined at the concentration given in the experimental section. Enantioselectivity and favored enantiomer are in parentheses. [b] Rate refers to formation of the substrate shown. [c] Measured rate at 0.5 mM methyl salicylate. The product, salicylic acid inhibits SABP2 (K_d = 90 nM[2]). Other researchers measured a faster rate of 27 min^{-1} when product inhibition does not slow the reaction.

Table S3. Rates and enantioselectivity of other nucleophilic additions and other hydrolyses catalyzed by modern and ancestral hydroxynitrile lyases and esterases.[a]

other eliminations



other hydrolyses



Enzymes	**Decarboxylase** Benzoylacetic acid	**Michael Addition** 3-(2-Nitro-1-phenyl ethyl)pentane-2,4-dione[d]	**Lactamase** 2-Azabicyl-[2.2.1]hept-5-en-3-one	**C–C hydrolase** 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoic acid
*Hb*HNL	<0.0001	<0.0001	<0.0001	<0.006
*Me*HNL	<0.0001	<0.0001	<0.0001	<0.08
HNL1-ML	<0.0001	<0.0001	<0.0001	<0.2
HNL1-NJ	0.015 ±0.0008	0.0002 ±0.0001 (3.4, *S*)	<0.0001	0.00051 ± 0.00005
HNL1	<0.0001	<0.0001	<0.0001	<0.001
EST3-ML	<0.0001	<0.0001	<0.0001	0.0012 ± 0.0002
EST3-NJ	<0.0005	0.00015 ±0.00006 (1.1, *R*)	0.0041 ± 0.00005 (66, 1*R*, 4*S*)	0.0056 ± 0.0005
EST3	<0.0002	<0.0001	<0.0001	0.031 ± 0.003
EST2	0.0026±0.0003	<0.0001	0.0018±0.0001 (9.8, 1*R*, 4*S*)	<0.08
EST1	<0.0001	<0.0001	0.021 ± 0.002 (82, 1*R*,4*S*)	<0.005
SABP2	<0.0001	<0.0001	0.0027±0.0012 (40, 1*R*, 4*S*)	<0.002
*Rc*EST	<0.001	<0.001	<0.0005	<0.006
*Rs*EST	<0.0001	<0.0001	<0.0005	0.00035 ± 0.00003
*At*EST	<0.0001	<0.0001	<0.0001	<0.003

[a] Error limits are standard deviations from three measurements. Rates (min^{-1}) correspond to hydrolysis or cleavage of the substrate shown determined at the concentration given in the experimental section. Enantioselectivity and favored enantiomer are in parentheses. Red fill marks instances where no reaction was detected; the detection limits vary depending on the substrate and amount of enzyme available. [b] Rates are k_{cat} (min^{-1}) determined by steady state kinetics. [c] Enantioselectivity was measured by formation of the substrate shown. [d] Rate refers to formation of the substrate shown.

Table S4. Data (conversion and enantiomeric excess) to measure the enantioselectivity of modern and ancestral enzymes.[a]

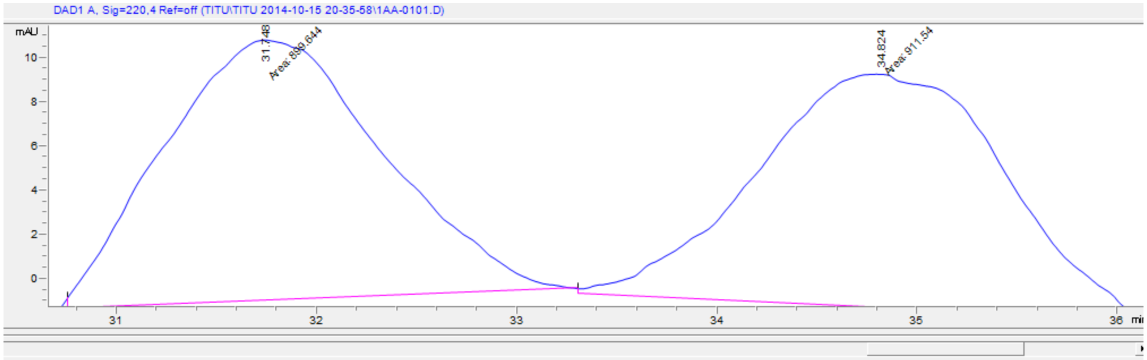
Enzymes	**Hydroxynitrile lyase** Mandelonitrile	**Henry Reaction** 2-Nitro-1-phenylethanol	**Michael addition** 3-(2-Nitro-1-phenyl ethyl)pentane-2,4-dione	**Lactamase** 2-Azabicyclo-[2.2.1]hept-5-en-3-one	**Lactonase** 5-phenyldihydrofuran-2(3H)-one	**Esterase** Methyl mandelate
*Hb*HNL	10, 99 (*S*)	63, 92 (*S*)[b]	<0.1	<0.1	<0.1	<0.1
*Me*HNL	45, 96 (*S*)	76, 5 (*S*)	<0.1	<0.1	<0.1	<0.1
HNL1-ML	55, 60 (*S*)	85, 83 (*S*)	<0.1	<0.1	<0.1	<0.1
HNL 1-NJ	72, 95 (*S*)	98, 95 (*S*)	19, 56 (*S*)	<0.1	66, 99 (*S*)	<0.1
HNL 1	52, 95 (*S*)	91, 96 (*S*)	<0.1	<0.1	<0.1	<0.1
EST3-ML	18, 77 (*R*)	<0.1	<0.1	<0.1	<0.1	<0.1
EST3-NJ	<0.1	<0.1	11.6 (*R*)	47, 78 (1*R*, 4*S*)	21, 30 (*R*)	11, <5
EST3	<0.1	<0.01	<0.1	<0.1	<0.1	<0.1
EST 2	20, 39 (*S*)	<0.1	<0.1	37, 59 (1*R*, 4*S*)	34, 55 (*R*)	50, 10 (*S*)
EST 1	15, 36 (*S*)	<0.1	<0.1	52, 96 (1*R*, 4*S*)	24,34 (*R*)	13, <5
SABP2	<0.1	<0.1	<0.1	51, 92 (1*R*, 4*S*)	39, <5	10, <5
Rc EST	<0.1	<0.1	<0.1	<0.1	<0.1	5, <5
Rs EST	<0.1	<0.1	<0.1	<0.1	68, 99 (*S*)	12, 6 (*R*)
*At*EST	86, 99 (*R*)	96, 88 (*R*)	<0.1	<0.1	15, 24 (*R*)	<0.1

[a] The first number indicates the conversion in % and the second the enantiomeric excess in % of the product indicated for the first three reactions (nucleophilic additions) and of the unreacted substrate indicated for the last three reactions (hydrolyses). (R) or (S) indicates the configuration of the favored enantiomer. If the measured enantiomeric excess was ≤5% ee, the reaction is considered not enantioselective. [b]From reference 5.

Figure S3. Representative HPLC chromatograms to measure enantioselectivity of modern and ancestral enzymes

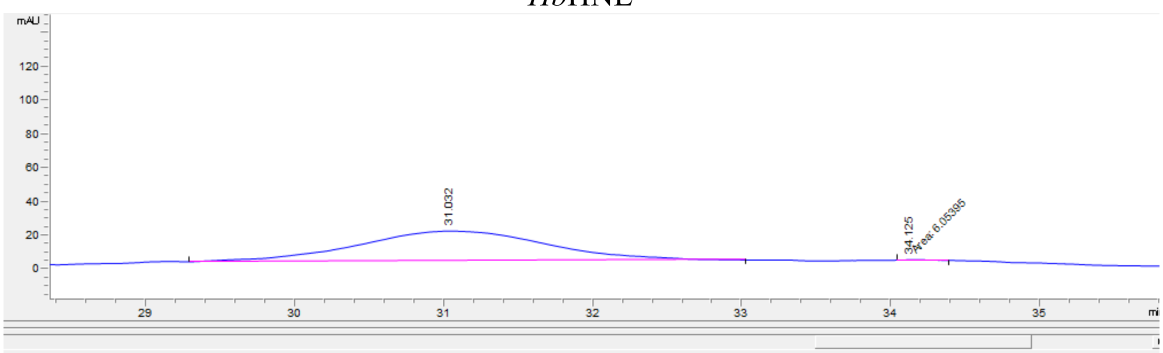
A: Formation of mandelonitrile (Chiralcel OD-H, hexane: isopropanol. 98:2)

Racemic mandelonitrile



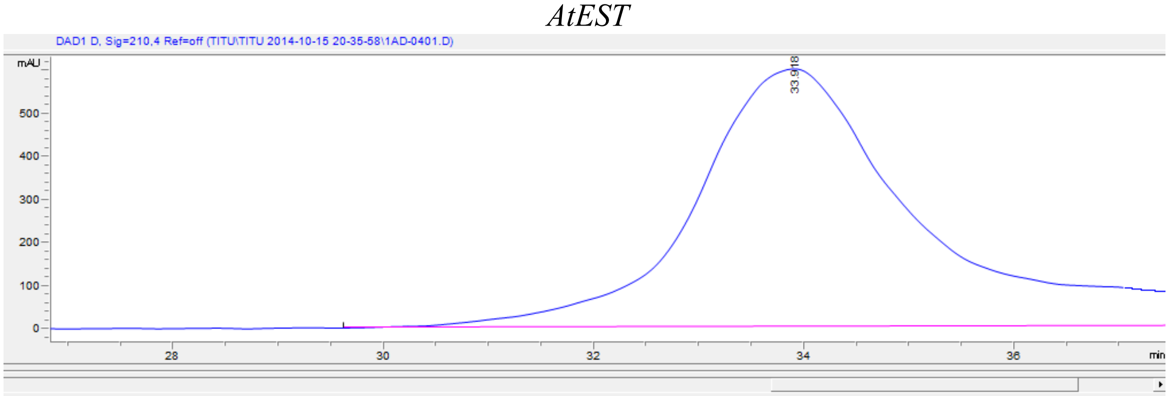
#	Time	Area	Height	Width	Area%	Symmetry
1	31.748	899.6	11.8	1.2674	49.672	0.874
2	34.824	911.5	10.6	1.4336	50.328	0.932

*Hb*HNL



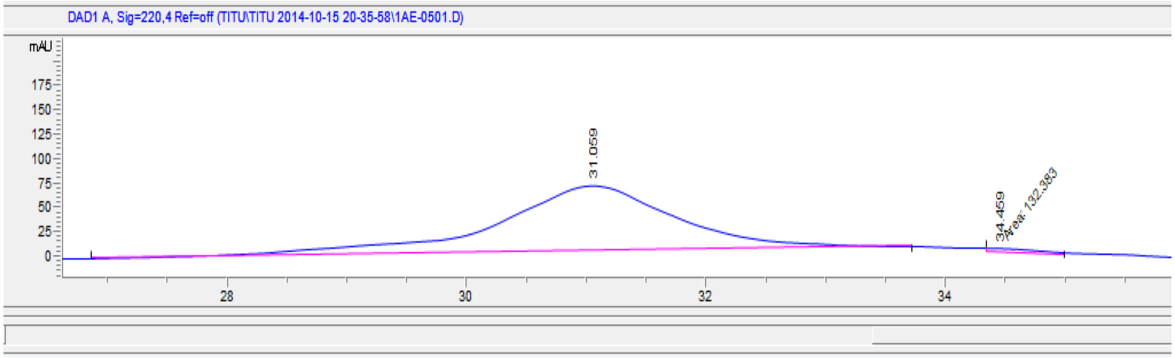
#	Time	Area	Height	Width	Area%	Symmetry
1	31.032	1573.6	17.7	1.0543	99.617	0.923
2	34.125	6.1	4.5E-1	0.2256	0.383	0.39

*At*EST



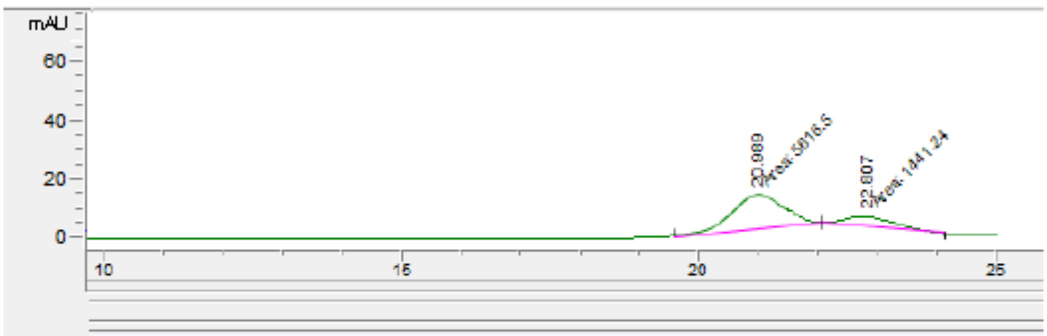
#	Time	Area	Height	Width	Area%	Symmetry
1	33.918	91582.6	596.9	2.0461	100.000	0.696

*Me*HNL



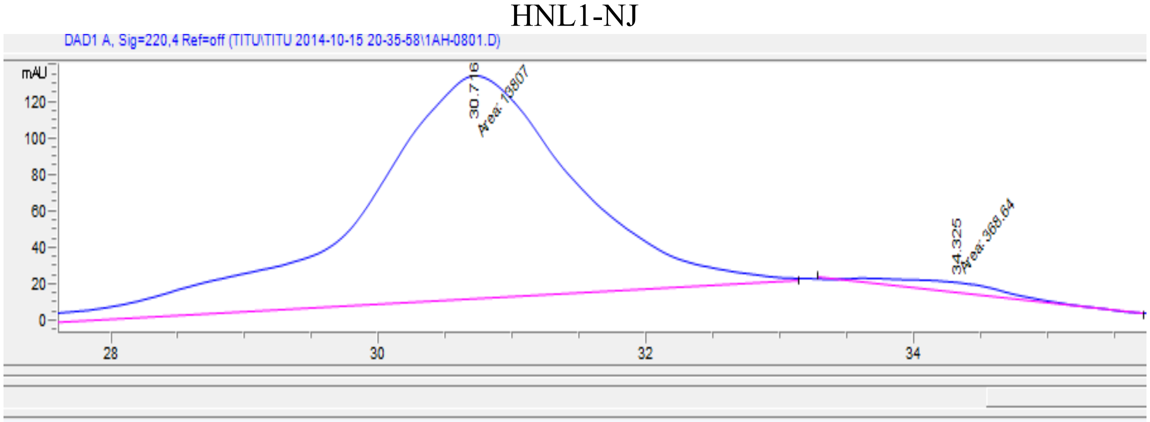
#	Time	Area	Height	Width	Area%	Symmetry
1	31.059	7110.5	67	1.354	98.172	1.197
2	34.459	132.4	4.2	0.522	1.828	0

HNL1-ML



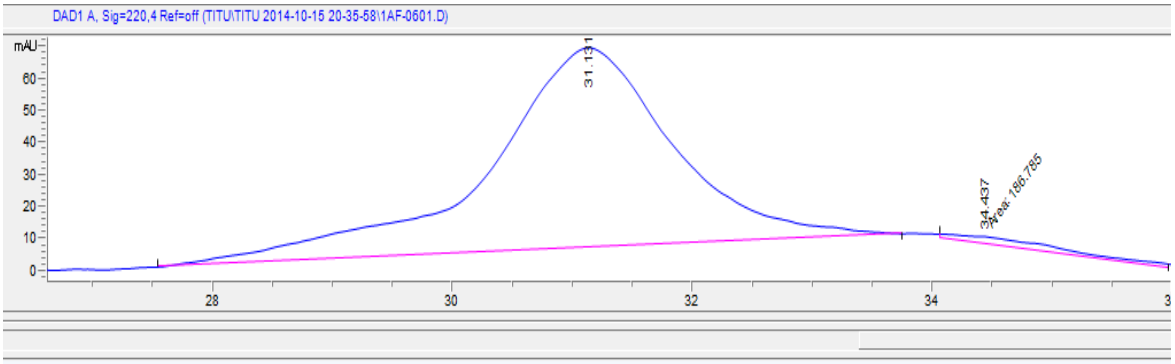
#	Time	Area	Height	Width	Area%
1	20.989	5616.5	93.2	1.0044	79.579
2	22.807	1441.2	24.9	0.9642	20.421

HNL1-NJ



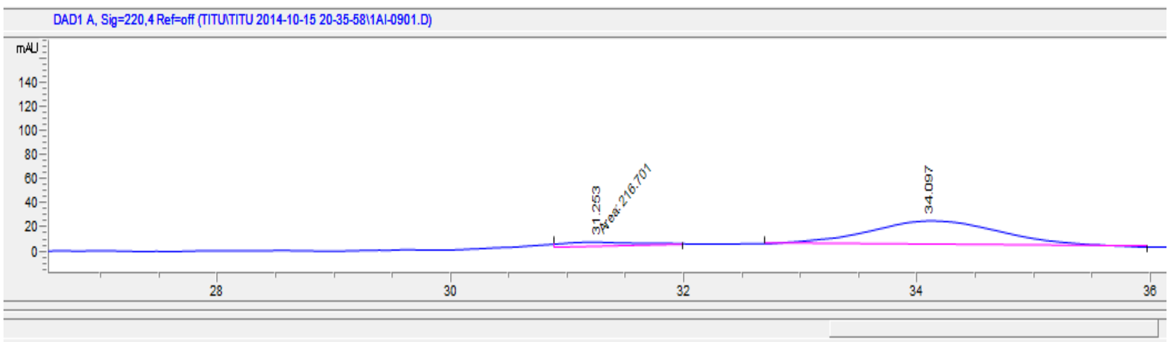
#	Time	Area	Height	Width	Area%	Symmetry
1	30.716	13807	122.5	1.879	97.399	1.171
2	34.325	368.6	5.8	1.0652	2.601	3.23E-2

HNL1



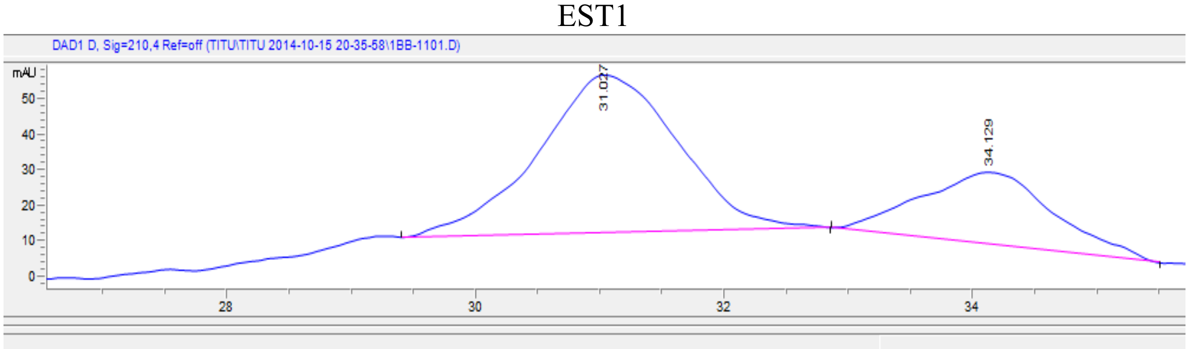
#	Time	Area	Height	Width	Area%	Symmetry
1	31.131	6631.2	62.1	1.352	97.260	1.192
2	34.437	186.8	2.4	1.2992	2.740	0.239

EST3-ML



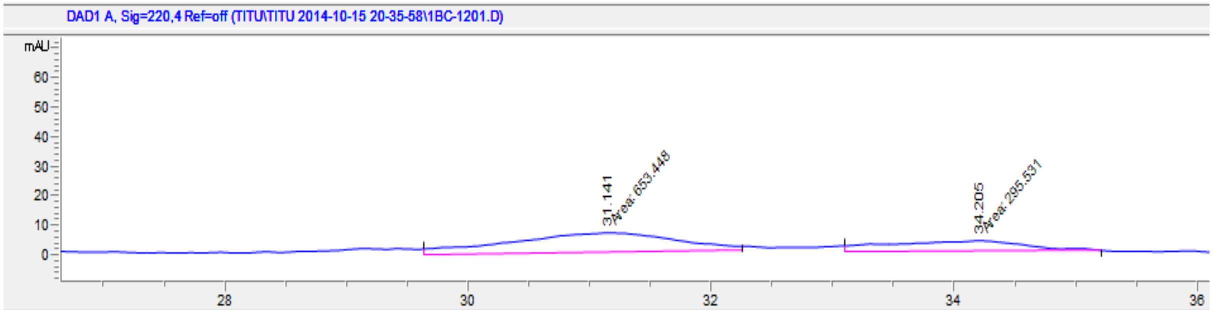
#	Time	Area	Height	Width	Area%	Symmetry
1	31.253	216.7	4.4	0.8168	11.551	0.767
2	34.097	1659.4	20.2	0.9729	88.449	0.799

EST1



#	Time	Area	Height	Width	Area%	Symmetry
1	31.027	3469.8	44.3	1.0101	69.712	0.9
2	34.129	1507.5	20.2	0.8969	30.288	1.016

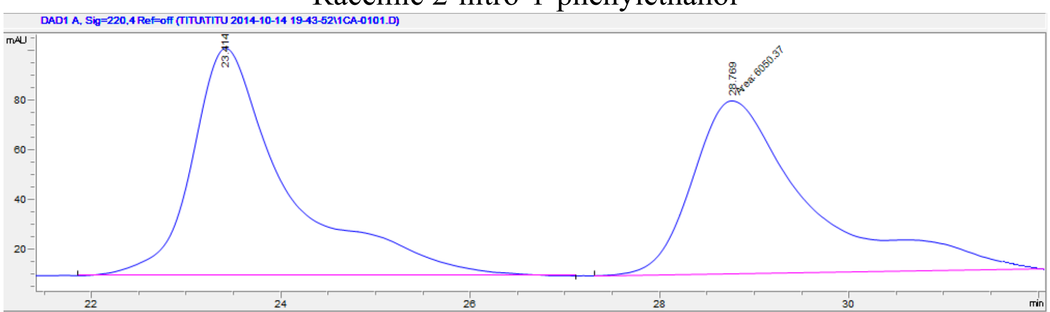
EST2



#	Time	Area	Height	Width	Area%	Symmetry
1	31.141	653.4	6.6	1.6396	68.858	1.452
2	34.205	295.5	3.7	1.3358	31.142	2.032

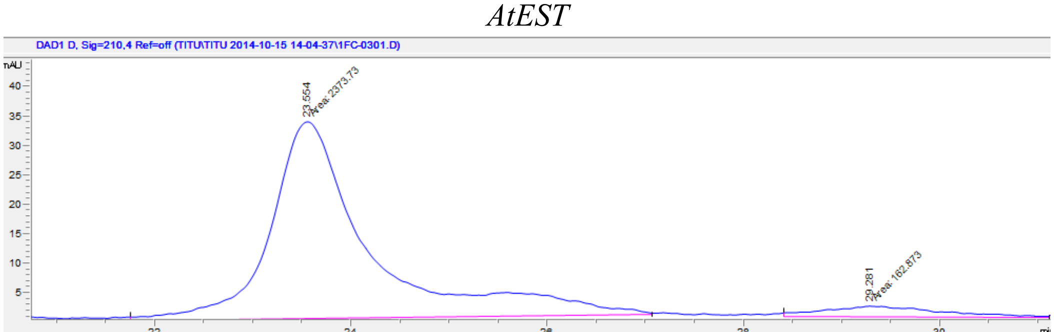
B: Addition of nitromethane to benzaldehyde (Henry reaction) (Chiralcel OD-H, hexane: isopropanol 95:5)

Racemic 2-nitro-1-phenylethanol



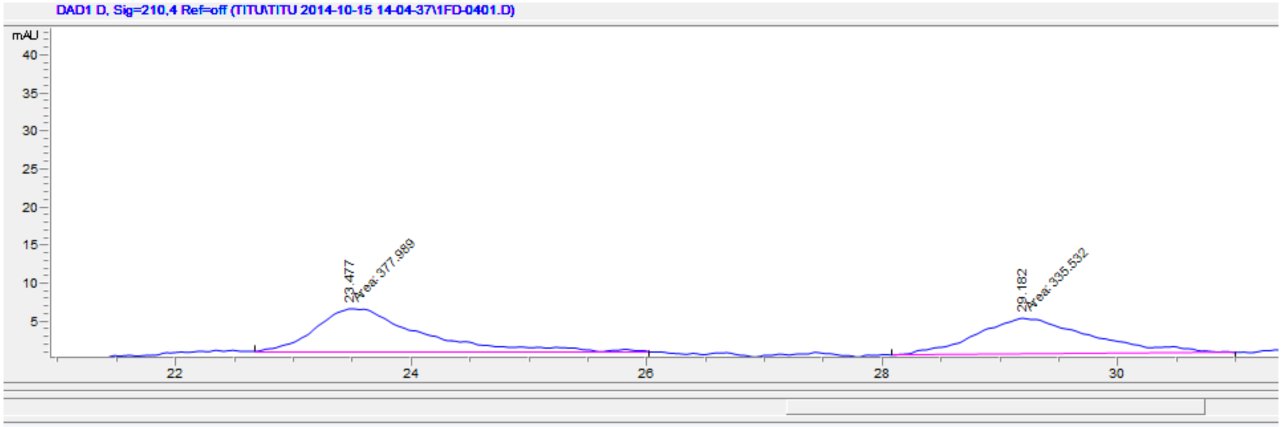
#	Time	Area	Height	Width	Area%	Symmetry
1	23.414	6494.6	91.5	0.9937	51.770	0.502
2	28.769	6050.4	69.9	1.4431	48.230	0.498

AtEST



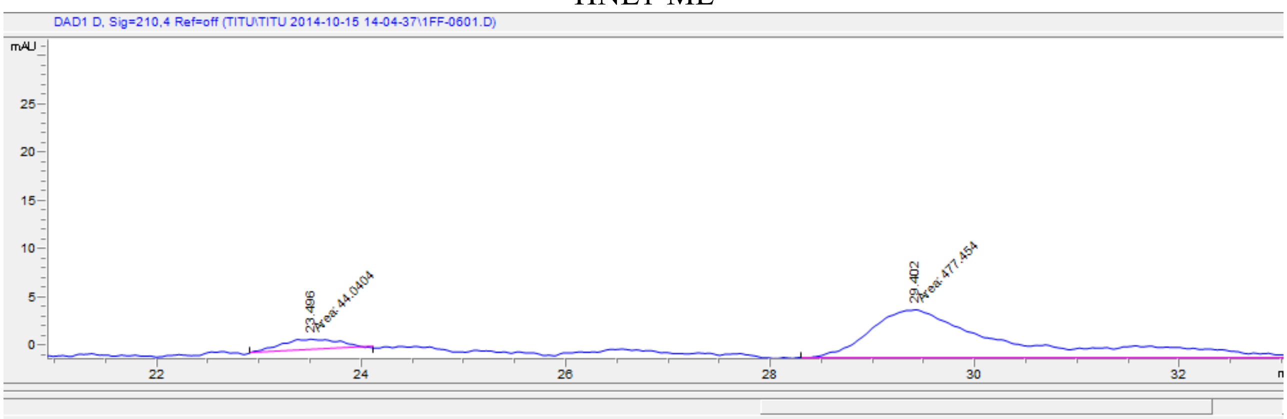
#	Time	Area	Height	Width	Area%	Symmetry
1	23.554	2373.7	33.4	1.1845	93.579	0.593
2	29.281	162.9	1.9	1.4574	6.421	0.593

*Me*HNL



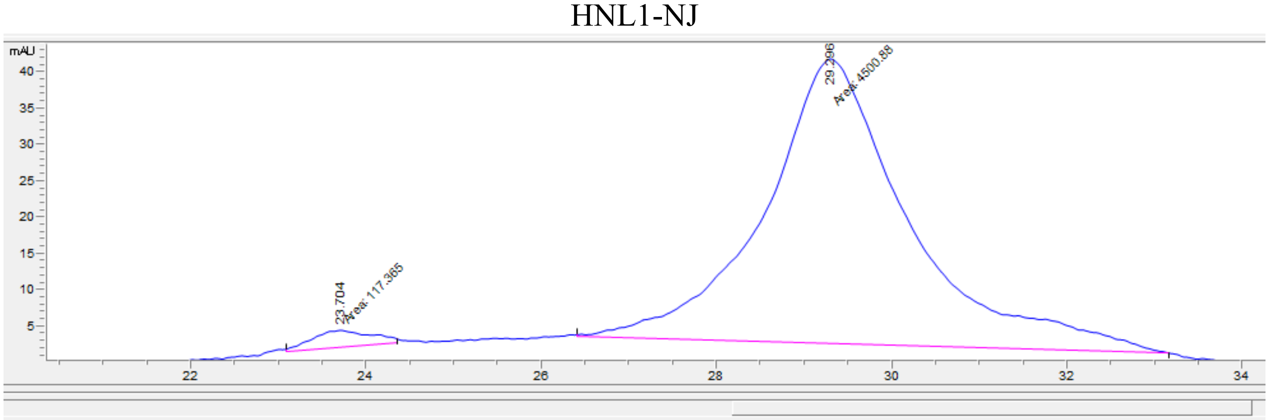
#	Time	Area	Height	Width	Area%	Symmetry
1	23.477	378	5.7	1.0966	52.975	0.474
2	29.182	335.5	4.8	1.1705	47.025	0.681

HNL1-ML



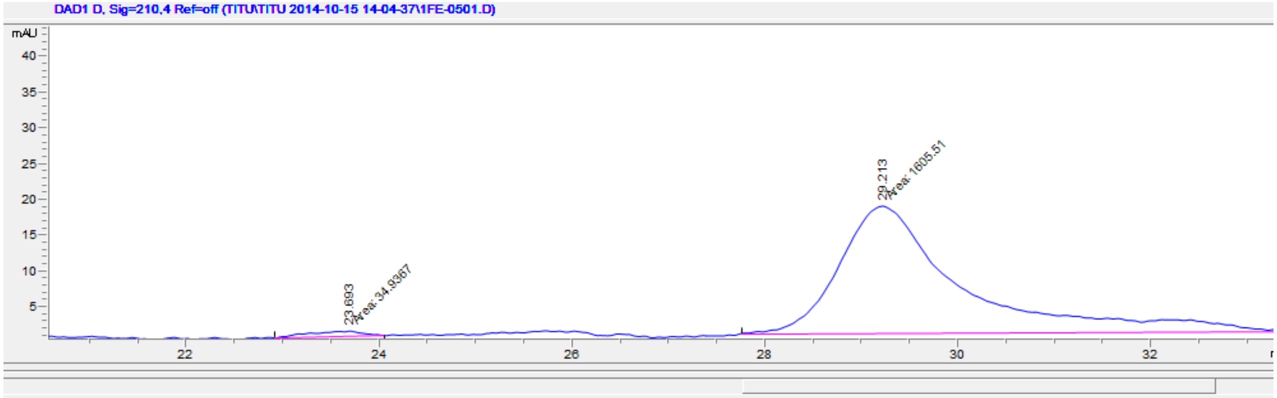
#	Time	Area	Height	Width	Area%	Symmetry
1	23.496	44	1.1	0.6678	8.445	1.14
2	29.402	477.5	5	1.5949	91.555	0.459

HNL1-NJ



#	Time	Area	Height	Width	Area%	Symmetry
1	23.704	117.4	2.4	0.8115	2.541	0.986
2	29.296	4500.9	39.3	1.9103	97.459	0.834

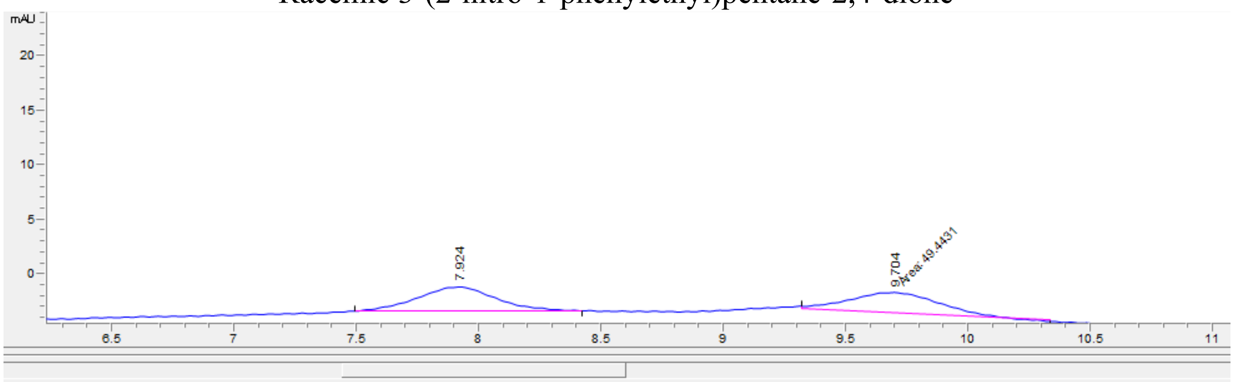
HNL 1



#	Time	Area	Height	Width	Area%	Symmetry
1	23.693	34.9	8E-1	0.7234	2.130	2.986
2	29.213	1605.5	17.9	1.4969	97.870	0.535

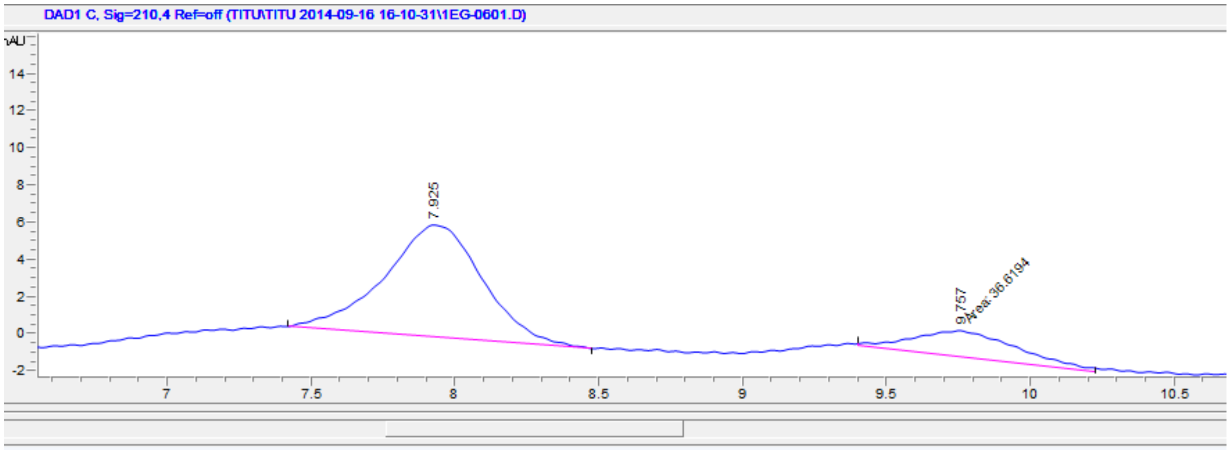
C. Addition of acetyl acetone to trans-β-nitrostyrene (Michael addition) (Chiralcel OJ-R, acetonitrile: H_2O, 40:60)

Racemic 3-(2-nitro-1-phenylethyl)pentane-2,4-dione



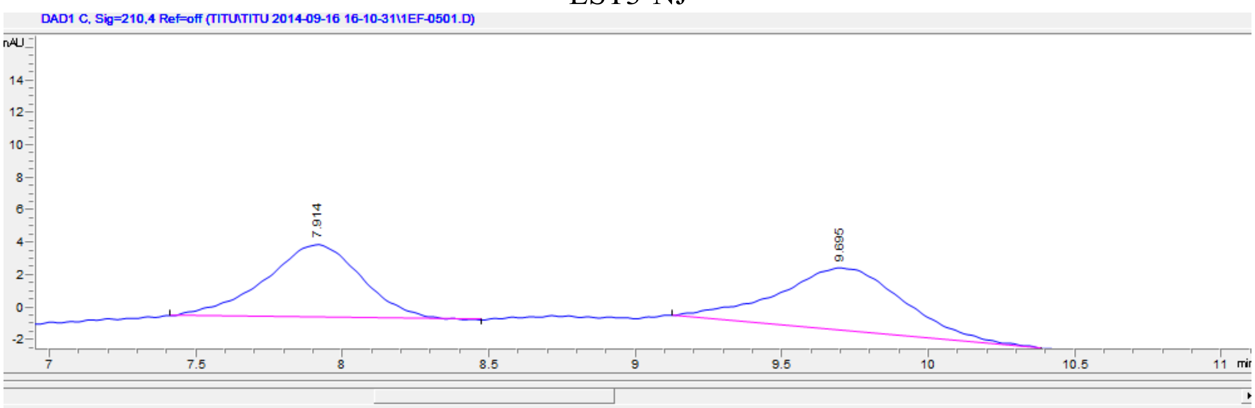
#	Time	Area	Height	Width	Area%	Symmetry
1	7.924	49.8	2.2	0.2757	50.202	1.16
2	9.704	49.4	1.9	0.4336	49.798	0.887

HNL1-NJ



#	Time	Area	Height	Width	Area%	Symmetry
1	7.925	143.7	6	0.2958	79.697	1.004
2	9.757	36.6	1.4	0.4265	20.303	0.707

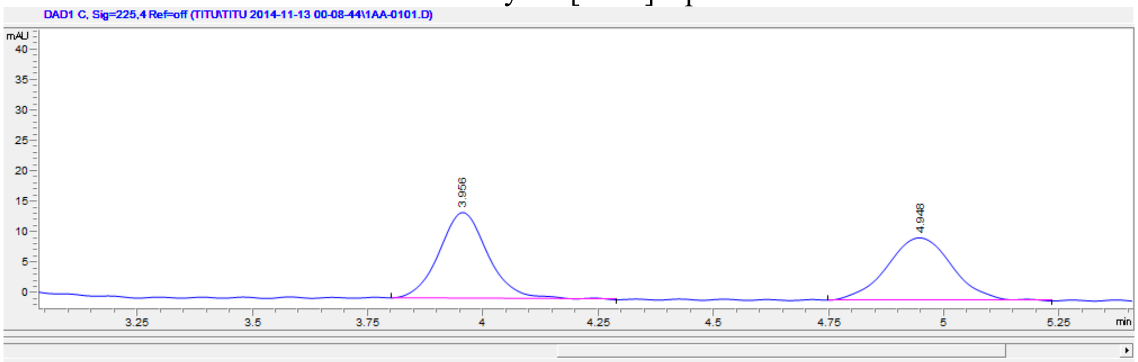
EST3-NJ



#	Time	Area	Height	Width	Area%	Symmetry
1	7.914	104.4	4.5	0.3051	47.224	1.164
2	9.695	116.7	3.9	0.364	52.776	0.913

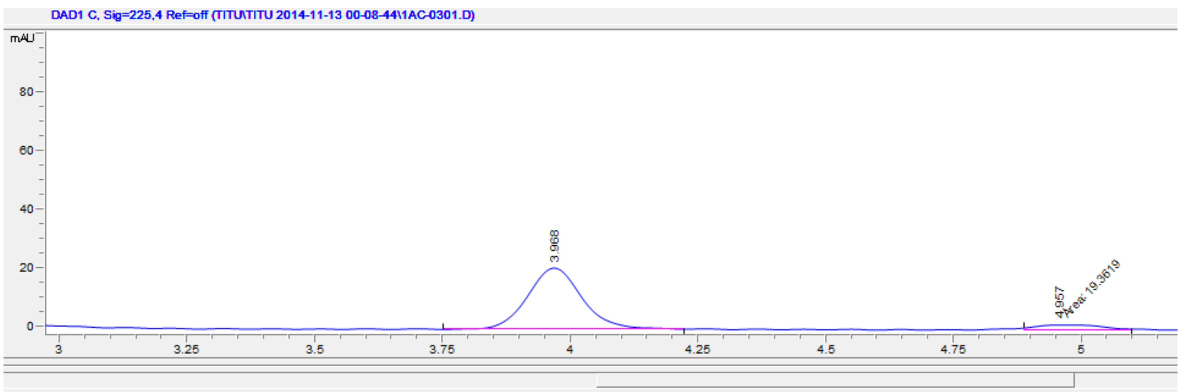
D: Hydrolysis of 2-azabicyclo[2.2.1]hept-5-en-3-one (lactamase) (Chiralcel AS-RH, acetonitrile : H_2O + 0.1% formic acid, 20:80)

Racemic 2-azabicyclo-[2.2.1]hept-5-en-3-one



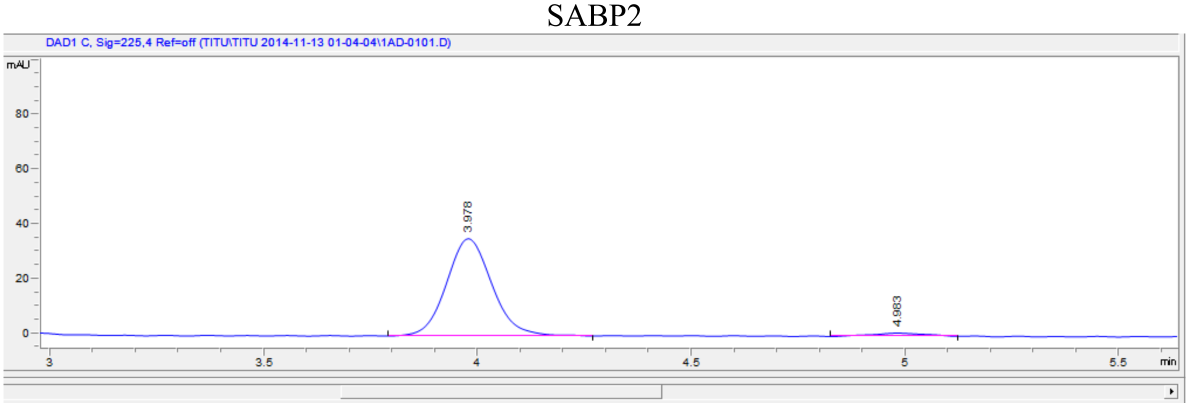
#	Time	Area	Height	Width	Area%	Symmetry
1	3.956	104.8	14	0.1131	50.722	0.869
2	4.948	101.8	10.3	0.1538	49.278	0.948

EST3-NJ



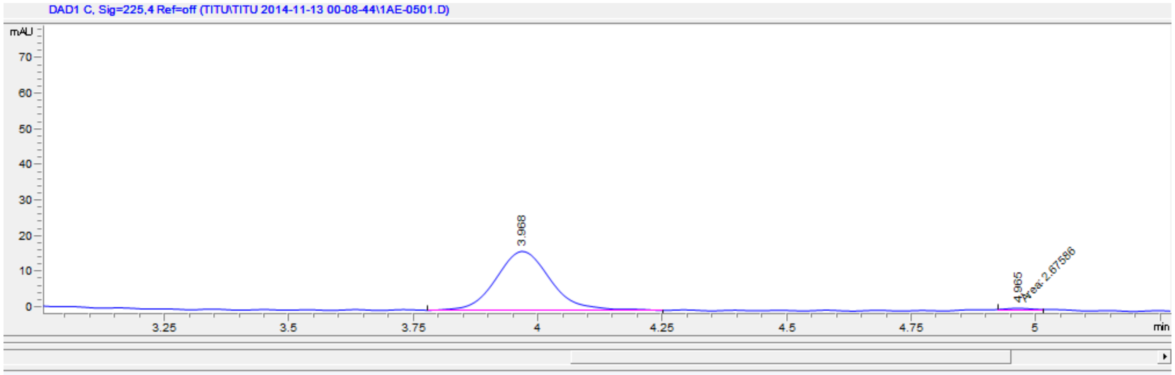
#	Time	Area	Height	Width	Area%	Symmetry
1	3.968	158.4	21	0.1139	89.110	0.904
2	4.957	19.4	2	0.1597	10.890	0.495

SABP2



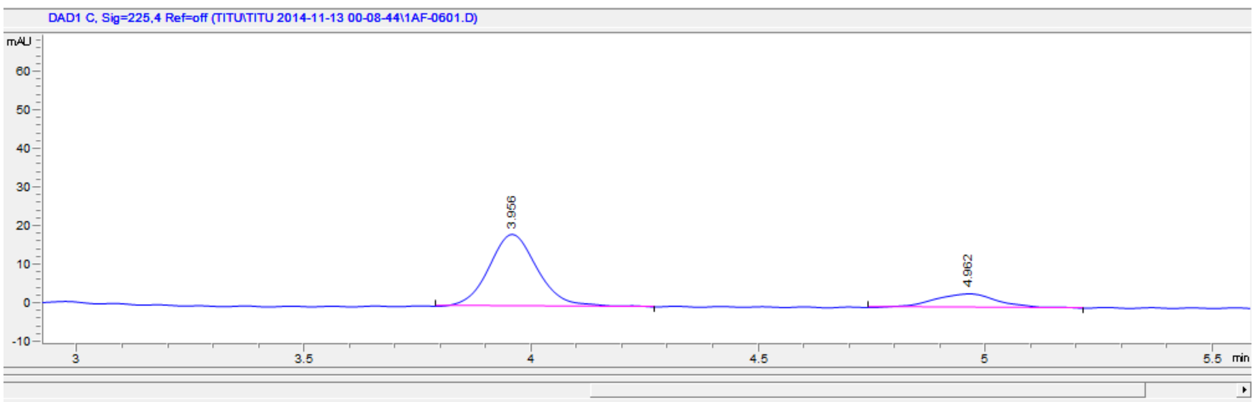
#	Time	Area	Height	Width	Area%	Symmetry
1	3.978	264.5	35.7	0.1145	95.997	0.878
2	4.983	11	1.2	0.1234	4.003	0.996

EST1



#	Time	Area	Height	Width	Area%	Symmetry
1	3.968	126.6	16.6	0.1172	97.929	0.893
2	4.965	2.7	6.4E-1	0.0702	2.071	0.744

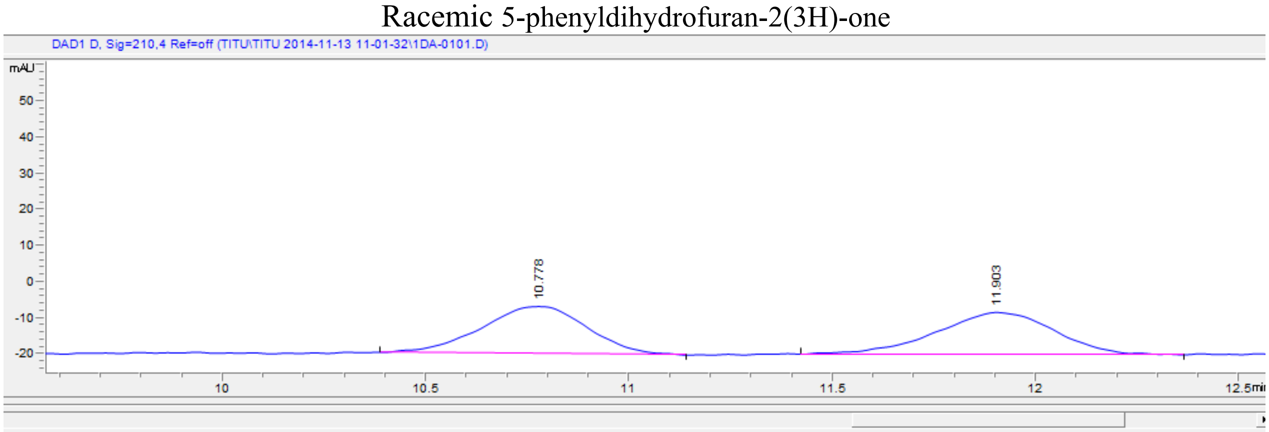
EST2



#	Time	Area	Height	Width	Area%	Symmetry
1	3.956	140	18.7	0.1134	79.107	0.859
2	4.962	37	3.6	0.1609	20.893	1.144

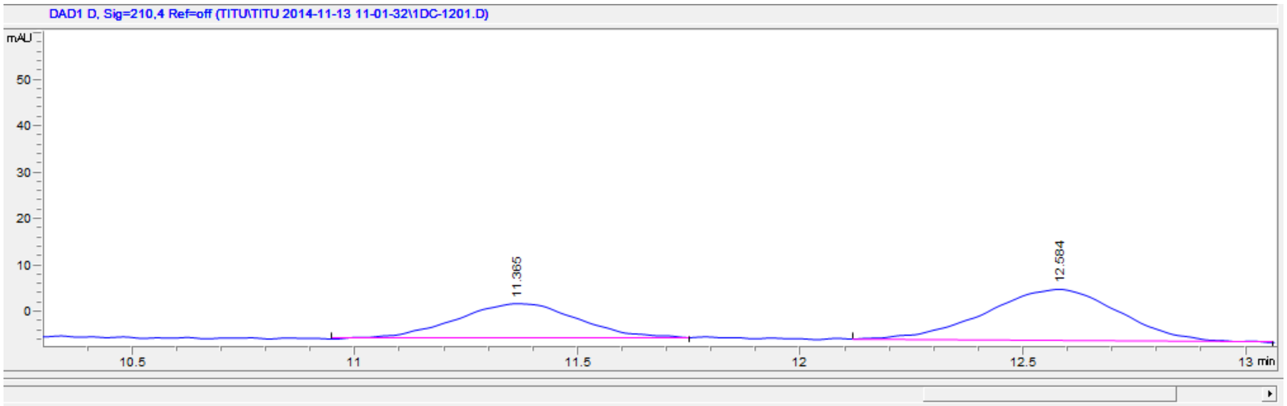
E. Hydrolysis of 5-phenyldihydrofuran-2(3H)-one (lactonase) (Chiralcel AS-RH, acetonitrile: H_2O + 0.1% formic acid, 35:65)

Racemic 5-phenyldihydrofuran-2(3H)-one



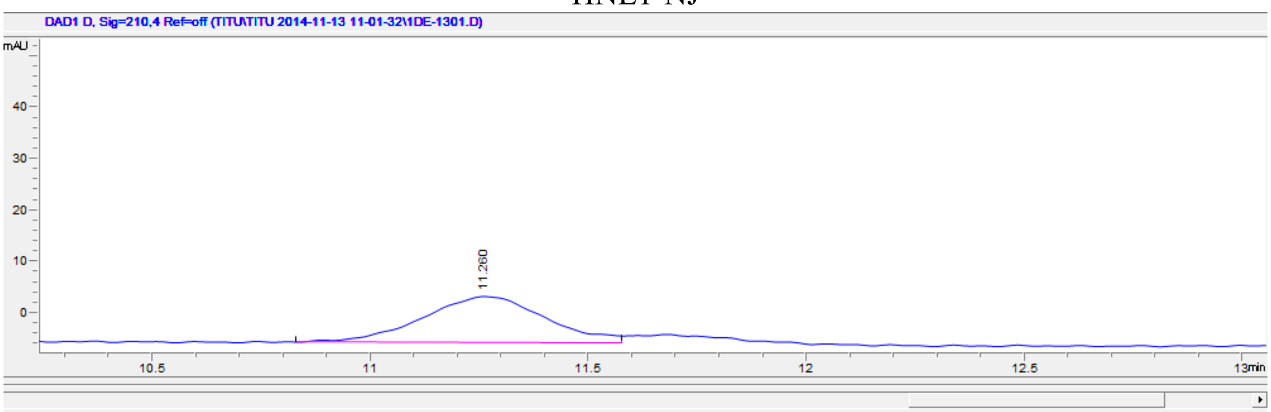
#	Time	Area	Height	Width	Area%	Symmetry
1	10.778	237.5	13.1	0.2861	49.473	1.149
2	11.903	242.6	11.7	0.301	50.527	1.093

AtEST



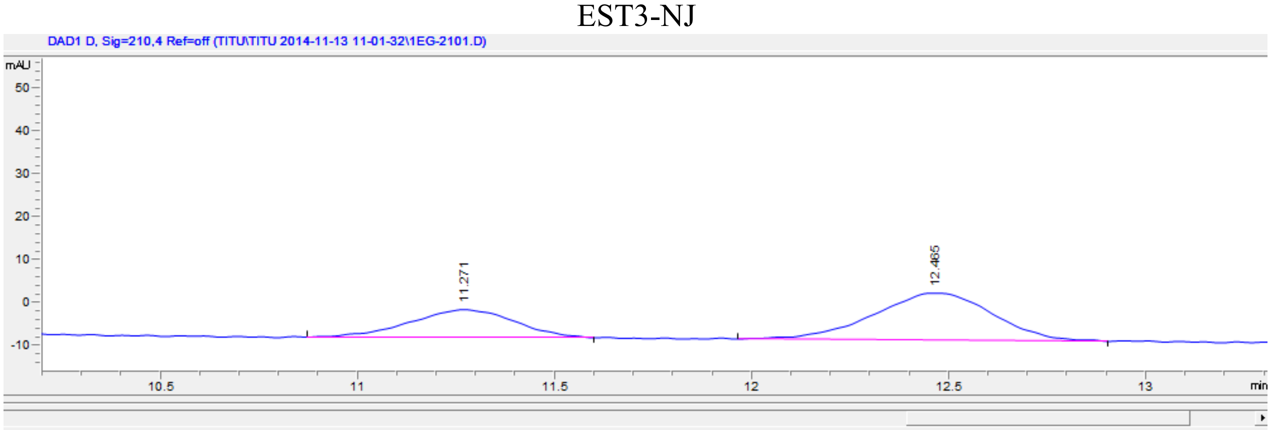
#	Time	Area	Height	Width	Area%	Symmetry
1	11.365	146.1	7.5	0.2609	38.070	1.037
2	12.584	237.6	11.2	0.2857	61.930	1.186

HNL1-NJ



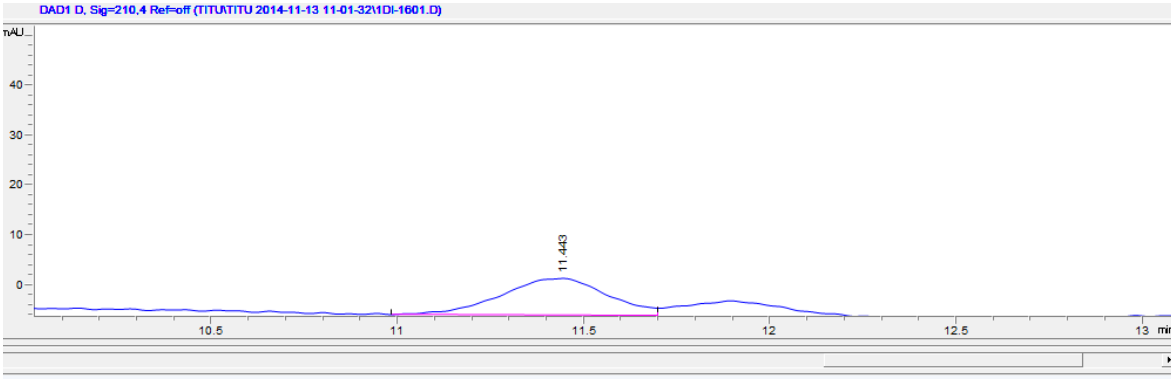
#	Time	Area	Height	Width	Area%	Symmetry
1	11.26	178	9	0.2662	100.000	0.986

EST3-NJ



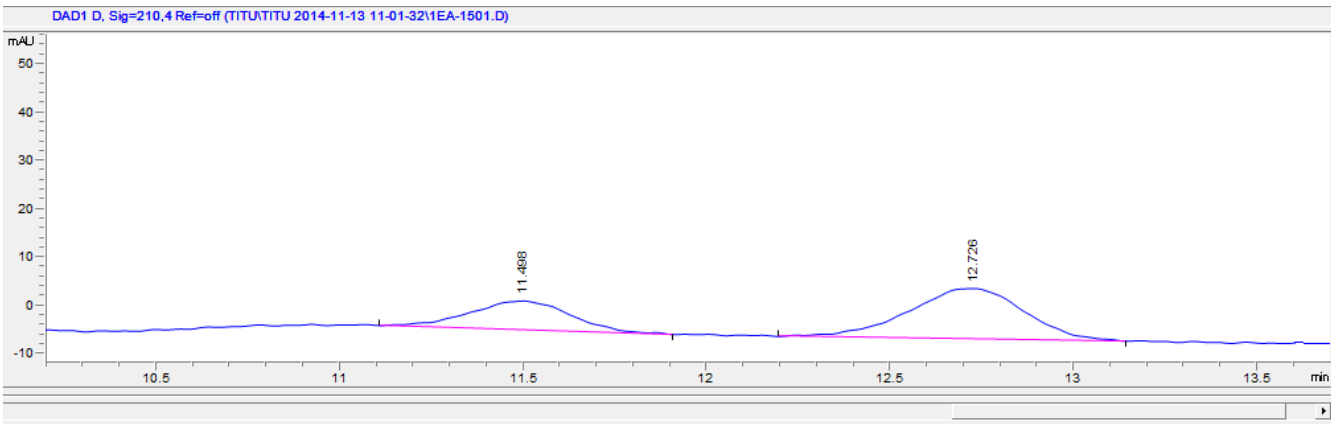
#	Time	Area	Height	Width	Area%	Symmetry
1	11.271	127.1	6.7	0.2398	35.189	1.056
2	12.465	234.1	11	0.2854	64.811	1.046

*Rs*EST



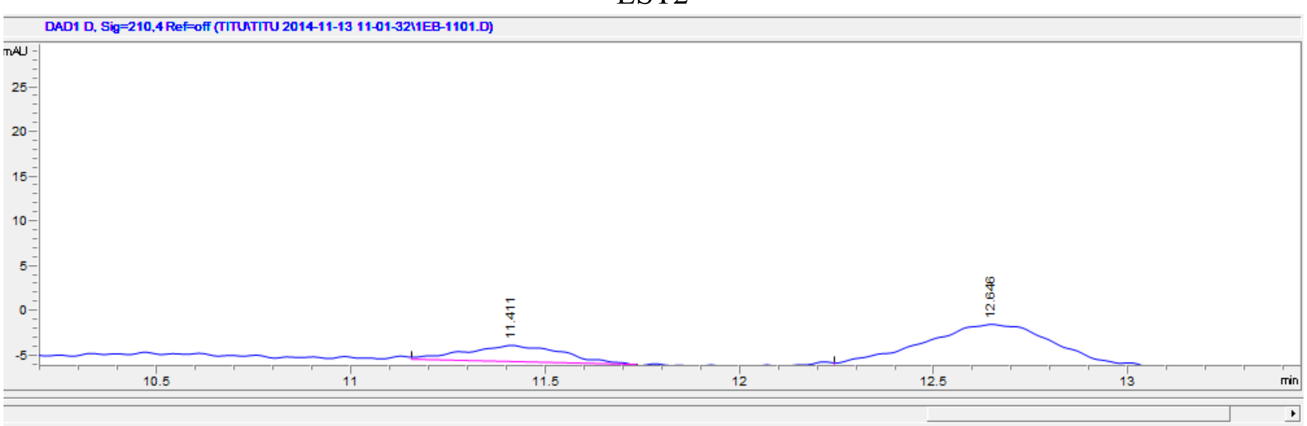
#	Time	Area	Height	Width	Area%	Symmetry
1	11.443	148.7	7.4	0.2514	100.000	1.346

EST1



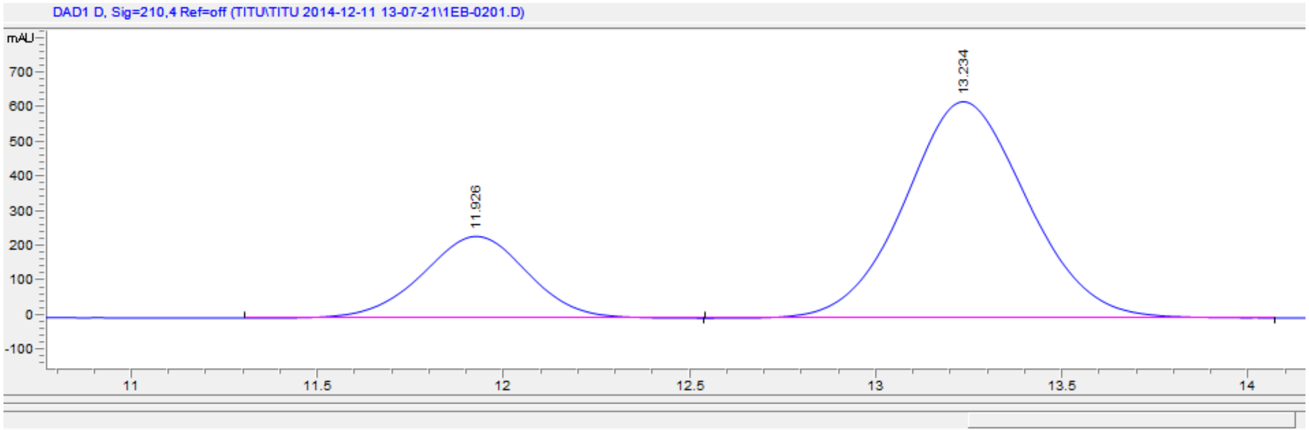
#	Time	Area	Height	Width	Area%	Symmetry
1	11.498	116.1	6	0.2408	33.322	1.072
2	12.726	232.3	10.5	0.2934	66.678	1.283

EST2



#	Time	Area	Height	Width	Area%	Symmetry
1	11.411	34.3	1.9	0.2228	22.433	0.804
2	12.646	118.7	4.8	0.2992	77.567	1.126

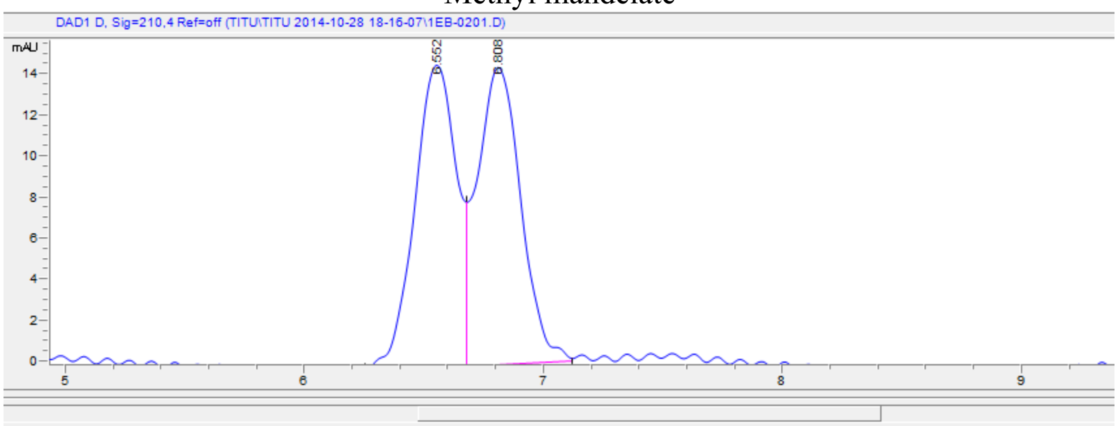
Porcine liver estearase (PLE) mediated hydrolysis of 5-phenyldihydrofuran-2(3H)-one to assign the first eluting peak as the fast-reacting (+)-enantiomer.



#	Time	Area	Height	Width	Area%	Symmetry
1	11.926	4677.6	236	0.3012	24.540	1.031
2	13.234	14383.3	625	0.3565	75.460	0.898

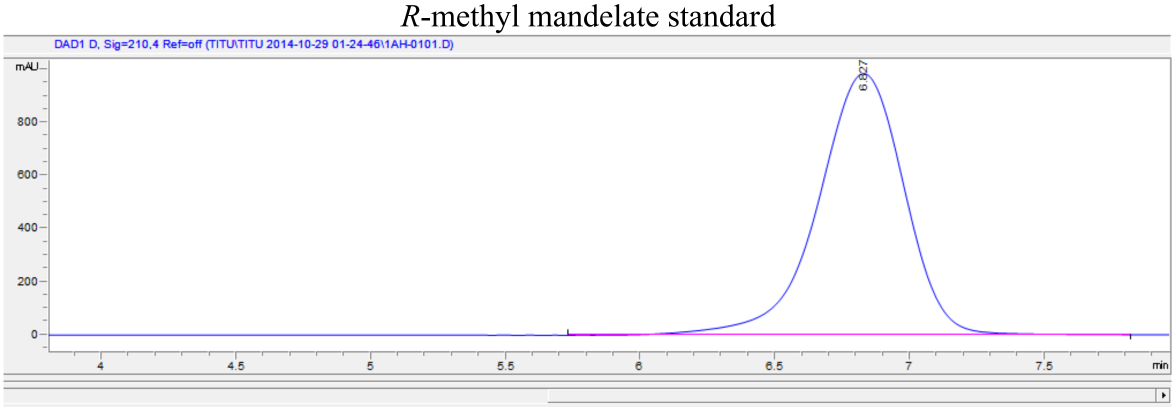
F: Hydrolysis of methyl mandelate (Chiralcel AS-RH, acetonitrile : H_2O + 0.1% trifluroacetic acid, 30:70)

Methyl mandelate



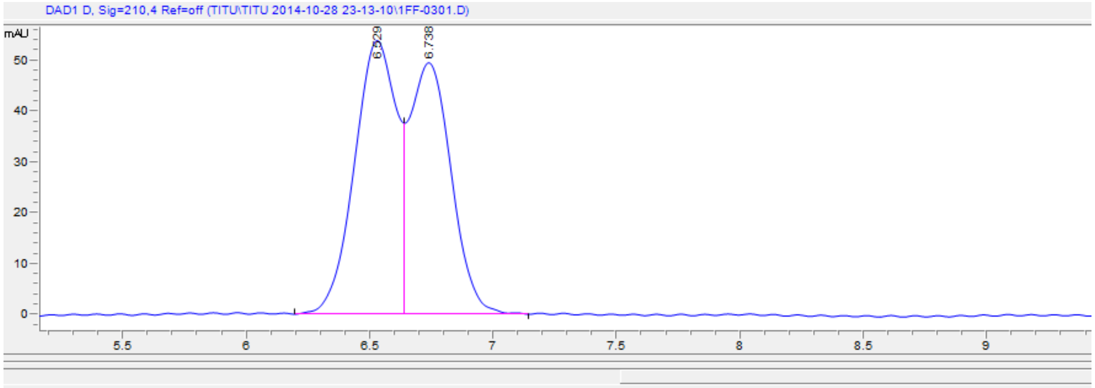
#	Time	Area	Height	Width	Area%	Symmetry
1	6.552	180.1	14.7	0.1869	49.496	1.145
2	6.808	183.8	14.5	0.1903	50.504	0.86

R-methyl mandelate standard



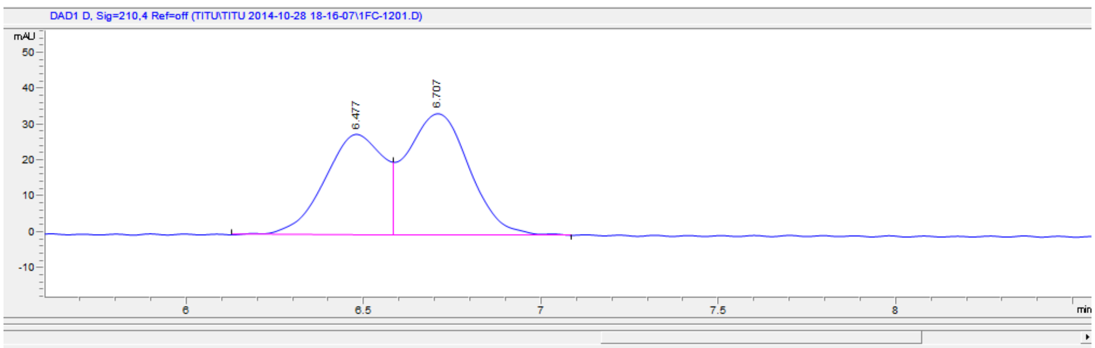
#	Time	Area	Height	Width	Area%	Symmetry
1	6.827	21905.5	984.6	0.3456	100.000	1.138

*Rs*EST



#	Time	Area	Height	Width	Area%	Symmetry
1	6.529	646.1	53.7	0.1803	52.926	1.17
2	6.738	574.6	49.4	0.1758	47.074	0.763

EST2



#	Time	Area	Height	Width	Area%	Symmetry
1	6.477	333.4	28.2	0.174	44.582	1.191
2	6.707	414.4	34	0.1844	55.418	0.899

Table S5. Categorical summary of data in Table S1.

	EC 4		**EC 3**	
Enzyme	**Y**	**N**	**Y**	**N**
*Hb*HNL	**6**	**0**	**1**	**5**
*Me*HNL	**6**	**0**	**0**	**6**
HNL1Mean	**6**	**0**	**4**	**2**
EST3Mean	**1**	**5**	**3**	**3**
EST2	**2**	**4**	**6**	**0**
EST1	**2**	**4**	**6**	**0**
SABP2	**0**	**6**	**6**	**0**
*Rc*EST	**0**	**6**	**5**	**1**
*Rs*EST	**0**	**6**	**6**	**0**
AtHNL	**2**	**4**	**4**	**2**

Table S6. 2x2 Fisher's exact test the substrate and catalytic promiscuity of modern and ancestral enzymes.

	natural reaction		**unnatural reaction**	
Enzyme	yes	no	yes	no
modern	33	3	3	33
ancestral	21	9	9	15

natural (substrate promiscuity), P = 0.67
unnatural (catalytic promiscuity), P = 0.0085

Table S7. Categorical summary of data in Table S2

	non-selected reactions	
Enzyme	**Y**	**N**
*Hb*HNL	**0**	**4**
*Me*HNL	**0**	**4**
HNL1Mean	**0**	**4**
EST3Mean	**1**	**3**
EST2	**2**	**2**
EST1	**1**	**3**
SABP2	**1**	**3**
*Rc*EST	**0**	**4**
*Rs*EST	**1**	**3**
AtHNL	**0**	**4**

Table S8. 2x2 Fisher's exact test the substrate and catalytic promiscuity of modern and ancestral enzymes.

	non-selected reaction	
Enzyme	yes	no
modern	2	22
ancestral	6	10

ancestral enzymes are more likely than modern enzymes to catalyze a promiscuous, non-selected reaction, P = 0.042

References

1. Hillis, D. M.; Bull, J. J. *Syst. Biol.* **1993**, *42*, 182.

2. Padhi, S. K.; Fujii, R.; Legatt, G. A.; Fossum, S. L.; Berchtold, R.; Kazlauskas, R. J. *Chem. Biol.* **2010**, *17*, 863.

3. Nedrud, D. M.; Lin, H.; Lopez, G.; Padhi, S. K.; Legatt, G. A.; Kazlauskas, R. J. *Chem. Sci.* **2014**, *5*, 4265.

4. Forouhar, F.; Yang, Y.; Kumar, D.; Chen, Y.; Fridman, E.; Park, S. W.; Chiang, Y.; Acton, T. B.; Montelione, G. T.; Pichersky, E.; Klessig, D. F.; Tong, L. *Proc. Natl. Acad. Sci., U. S. A.* **2005**, *102*, 1773.

5. Purkarthofer, T.; Gruber, K.; Gruber-Khadjawi, M.; Waich, K.; Skranc, W.; Mink, D.; Griengl, H. *Angew. Chem. Intl. Ed.* **2006**, *45*, 3454.