

Electric supplementary information (ESI)

Monoxygenase-catalyzed regioselective hydroxylation for the synthesis of hydroxyequols

Takafumi Hashimoto,^a Daiki Nozawa,^a Katsuyuki Mukai,^b Akinobu Matsuyama,^b Kouji Kuramochi^a and Toshiki Furuya^{*a}

Experimental details

Chemicals

(*S*)-Equol and (*R*)-equol were purchased from Wako Pure Chemicals (Osaka, Japan). Tween 80 was purchased from MP Biomedicals (Illkirch, France). All other chemicals were of analytical grade.

Construction of expression plasmids

A BLAST search of the genome sequences was performed with the amino acid sequence of HpaB of *Pseudomonas aeruginosa* PAO1 (HpaB_{pa}). The genome sequence of *Photorhabdus luminescens* subsp. *laumondii* TTO1 contains three *hpaB* homologues, *hpaB*_{pl-1}, *hpaB*_{pl-2}, and *hpaB*_{pl-3} (Table S1). The genome sequence of *Rhodococcus opacus* B-4 contains three *hpaB* homologues, *hpaB*_{ro-1}, *hpaB*_{ro-2}, and *hpaB*_{ro-3} (Table S1). These genes were amplified by PCR and then inserted into the pETDuet-1 vector using the primers and restriction enzymes listed in Table S1. The *hpaB* gene of *Escherichia coli* BL21(DE3) (*hpaB*_{ec}) was also cloned (Table S1). The *hpaB*_{pa} gene of *P. aeruginosa* PAO1 was previously cloned to construct the pETDhpaB plasmid.¹ The *hpaC* gene of *P.*

aeruginosa PAO1 (*hpaC_{pa}*) was amplified from the previously constructed pETDhpaBC plasmid by PCR and then inserted into the pCDFDuet-1 vector using the primers and restriction enzymes listed in [Table S1](#).¹

Preparation of whole cells

The pETDuet-1 vector carrying each *hpaB* homologue and the pCDFDuet-1 vector carrying *hpaC_{pa}* were introduced into *E. coli* BL21 Star (DE3) cells (Invitrogen, Carlsbad, CA, USA). The transformed *E. coli* cells were cultivated at 30°C in LB medium containing (per liter) Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) (pH 7.0), supplemented with ampicillin (50 µg mL⁻¹) and streptomycin (50 µg mL⁻¹). After cultivation for 6 h (OD₆₀₀=0.8–1.0), isopropyl-β-D-thiogalactopyranoside (1 mM) was added to the medium and cultivation was continued for an additional 15 h at 25°C. Cells were harvested by centrifugation and were washed with potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10% v/v). These cells were used for whole-cell reactions.

The *hpaB_{pl-1}* gene was also co-expressed with the chaperonin GroEL and the cochaperonin GroES in *E. coli* cells. The pGro7 plasmid (Takara Bio, Tokyo, Japan) was used for the co-expression of GroEL and GroES, as described previously.² The pETDuet-1 vector carrying *hpaB_{pl-1}*, the pCDFDuet-1 vector carrying *hpaC_{pa}*, and pGro7 were introduced into *E. coli* BL21 Star (DE3) cells. The transformed *E. coli* cells were cultivated at 30°C in LB medium supplemented with ampicillin (50 µg mL⁻¹), streptomycin (50 µg mL⁻¹), chloramphenicol (30 µg mL⁻¹), and arabinose (4 mg mL⁻¹). After cultivation for 6 h (OD₆₀₀=0.8–1.0), isopropyl-β-D-thiogalactopyranoside (1 mM) was added to the medium and cultivation was continued for an additional 15 h at 15°C.

Reactions using whole cells

The reaction mixture (250 μ L) contained cells of transformed *E. coli* strain (50 g of wet cell weight per liter), (*S*)-equol or (*R*)-equol (10 mM), dimethylsulfoxide (4% v/v), Tween 80 (1.5% v/v), and potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10% v/v). The reactions were carried out at 30°C with vigorous shaking using a microtube shaker.

Reactions on a flask scale

The reaction was carried out in a 500-mL flask containing cells of transformed *E. coli* strain (collected from 400 mL culture broth), (*S*)-equol (5 or 10 mM) or (*R*)-equol (5 or 10 mM), dimethylsulfoxide (4% v/v), Tween 80 (1.5% v/v), and potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10% v/v) in a volume of 20 mL. The reactions were carried out at 30°C with reciprocal shaking using a flask shaker.

Product analysis

High-performance liquid chromatography (HPLC) analysis was performed using an LC-20 system (Shimadzu, Kyoto, Japan) with an XTerra MS C18 IS column (4.6 \times 20 mm; particle size, 3.5 μ m; Waters, Milford, MA, USA). The reaction mixture (250 μ L) was acidified by the addition of HCl (pH 2–3), and methanol (500 μ L) and water (250 μ L) were then added. The solution was vigorously shaken and centrifuged. The resulting supernatant (10 μ L) was injected into the HPLC system. Mobile phases A and B were composed of 0.1% formic acid in water and of methanol, respectively. The mobile phase B was programmed as follows: start ratio of 5%, held at 5% for 3 min, increased to 40% for 1 min, increased to 80% for 10 min by a linear gradient, increased to 100% for 1 min, and held at 100% for 3 min. The flow rate was 0.5 mL min⁻¹. Compounds

were detected spectrophotometrically at a wavelength of 220 nm. The amounts of (*S*)-equol and (*R*)-equol were calculated from standard calibration curves that were made using the compounds purchased from Wako Pure Chemicals. The reaction products, 3'- and 6-hydroxyequols, were purified using column chromatography. The reaction mixture was acidified by the addition of HCl (pH 2–3) and extracted with ethyl acetate. After evaporation of the extract, the resulting residue was applied to a silica gel column (Wakogel 60N, 38–100 μm , Wako Pure Chemicals) and eluted with hexane/ethyl acetate. After evaporation of the fractions containing the product, the resulting residue was then applied to a C18 column (Cosmosil 75C18-OPN, Nacalai Tesque) and eluted with methanol/water. The amounts of 3'- and 6-hydroxyequols were calculated from standard calibration curves that were made using the compounds isolated in this study. Mass analysis was performed using a Thermo Finnigan LCQ (Waltham, MA, USA) with an electrospray ionization, as described previously.³ Nuclear magnetic resonance (NMR) analysis was performed using a Bruker Spectrospin 400 (Billerica, MA, USA), as described previously.³

3'-Hydroxyequol: ¹H NMR (400 MHz, methanol-*d*₄): δ =2.75 (m, 2H, H-4), 2.90 (m, 1H, H-3), 3.79 (m, 1H, H-2), 4.10 (m, 1H, H-2), 6.13 (d, *J*=2.4 Hz, 1H, H-8), 6.22 (dd, *J*=8.2, 2.4 Hz, 1H, H-6), 6.50 (dd, *J*=8.1, 2.0 Hz, 1H, H-6'), 6.60 (d, *J*=2.0 Hz, 1H, H-2'), 6.63 (d, *J*=8.1 Hz, 1H, H-5'), 6.78 (d, *J*=8.2 Hz, 1H, H-5); ¹³C NMR (400 MHz, methanol-*d*₄): δ =33.1 (C-4), 39.6 (C-3), 72.3 (C-2), 103.8 (C-8), 109.1 (C-6), 114.6 (C-4a), 115.4 (C-2'), 116.5 (C-5'), 119.6 (C-6'), 131.2 (C-5), 134.7 (C-1'), 145.2 (C-4'), 146.5 (C-3'), 156.3 (C-8a), 157.6 (C-7); MS (ESI) (*m/z*): calculated for C₁₅H₁₃O₄ [M-H]⁻: 257.0814, found: 257.0815.

6-Hydroxyequol: ^1H NMR (400 MHz, methanol- d_4): δ =2.79 (m, 2H, H-4), 3.02 (m, 1H, H-3), 3.84 (m, 1H, H-2), 4.12 (m, 1H, H-2), 6.24 (s, 1H, H-8), 6.48 (s, 1H, H-5), 6.73 (dd, J =6.6, 2.0 Hz, 2H, H-3'), 7.06 (dd, J =6.6, 1.8 Hz, 2H, H-2'); ^{13}C NMR (400 MHz, methanol- d_4): δ =33.2 (C-4), 39.6 (C-3), 72.1 (C-2), 104.4 (C-8), 113.8 (C-4a), 116.4 (C-3'), 116.6 (C-5), 129.4 (C-2'), 134.1 (C-1'), 140.1 (C-6), 145.5 (C-7), 148.7 (C-8a), 157.3 (C-4'); MS (ESI) (m/z): calculated for $\text{C}_{15}\text{H}_{13}\text{O}_4$ [M-H] $^-$: 257.0814, found: 257.0816.

References

- 1 T. Furuya and K. Kino, *Appl. Microbiol. Biotechnol.*, 2014, **98**, 1145-11542.
- 2 T. Furuya, M. Miura and K. Kino, *ChemBioChem*, 2014, **15**, 2248-2254.
- 3 T. Furuya and K. Kino, *ChemSusChem*. 2008, **2**, 645-649.

Table S1 Genes used in this study and primer sequences for gene cloning.

Gene	Accession no. ^a	Primer sequence (5' to 3') ^b	Digestion ^c
<i>hpaB_{pl-1}</i>	CAE12541	GAATTC <u>CATG</u> T CAG AAAAACATGAAACAAAAAT (forward)	PciI
		CGCGGATCC T AGCGGTTGGAAGGCCAAAT (reverse)	BamHI
<i>hpaB_{pl-2}</i>	CAE13270	TT CATG AAACCAGAAGATTTTCGCGCCG (forward)	BspHI
		CGCGGATCC T TATTT CAG CAGCTTATCTAA (reverse)	BamHI
<i>hpaB_{pl-3}</i>	CAE16399	TT CATG AAACCAGAAAATTTACGTACCG (forward)	BspHI
		CGCGGATCC T ACTTTTAGTAATTTATCTAA (reverse)	BamHI
<i>hpaB_{ro-1}</i>	BAH50341	TTCC CATG ACCACCACCGAAAGCGCACCC (forward)	NdeI
		GCGCAAT G CTACTTGTACCGAAGTACGA (reverse)	MunI
<i>hpaB_{ro-2}</i>	BAH50488	TTCC CATG ACCACCACCGAAGCTGCCCCC (forward)	NdeI
		GCGCAAT G CTAGCTGCGGCCGAAGTAGGA (reverse)	MunI
<i>hpaB_{ro-3}</i>	BAH51988	TTCC CATG ACCACATCAGCTTTCGTCGAC (forward)	NdeI
		GCGCAAT G TCAGCTCCGGACGATCCGCAG (reverse)	MunI
<i>hpaB_{ec}</i>	CAQ34705	TT CATG AAACCAGAAGATTTCCGCGCCA (forward)	BspHI
		CGCGGATCC T TATTT CAG CAGCTTATCCAG (reverse)	BamHI
<i>hpaB_{pa}</i>	AAG07478	TT CATG AAACCCGAAGATTTCCGTGCCT (forward)	BspHI
		CGCGGATCC T CATTGGCGGATGCGATCGAG (reverse)	BamHI
<i>hpaC_{pa}</i>	AAG07479	TTCC CATG TCCCAGCTCGAACCCAGGCAG (forward)	NdeI
		TTCGGTAC C TCAGGCCGCCCGGGGGCA (reverse)	KpnI

^a NCBI accession numbers for protein are indicated.

^b Restriction sites are underlined, and initiation and termination codons indicated in bold.

^c See Experimental details.

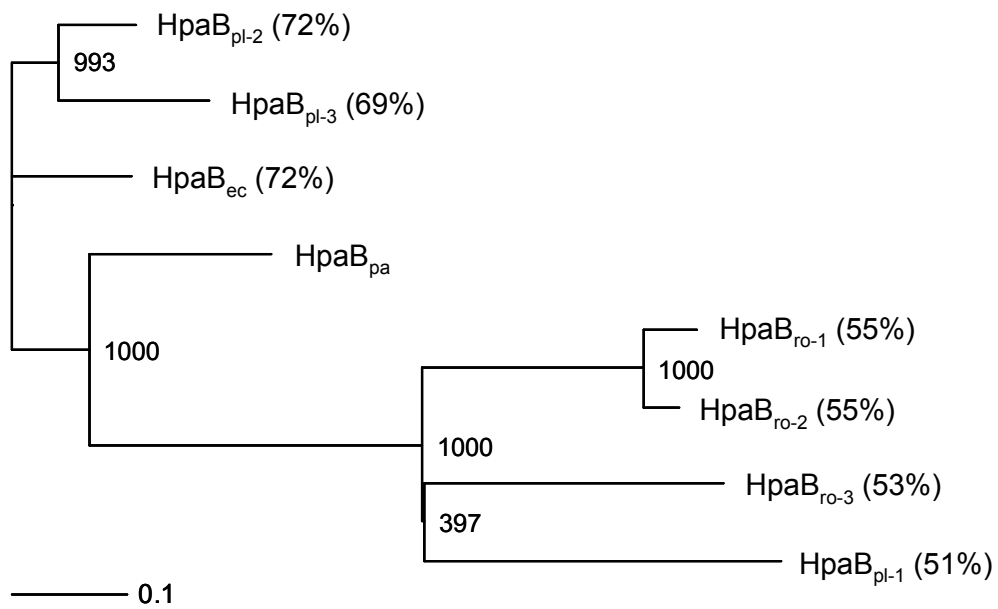


Fig. S1 Phylogenetic tree of eight HpaB enzymes. The HpaB enzymes analyzed are listed in Table S1. Amino acid identity between HpaB_{pa} and other HpaB enzymes are shown in parentheses.

HpaBtt 1 -----MARTGCEYLLEAKTRFPNLLWYKEMFE 27
HpaBpa 1 -----MKPFEDFRASATRFPTGCEYLLESLCD-DREIYLYGERVK 37
HpaBec 1 -----MKPFEDFRASATRFPTGCEYLLESLCD-DREIYLYGERVK 37
HpaBpl-1 1 -----MSENMKQKSSNEGNIRTRRPLTGTGELDQDND-GREIWAYGERFH 44
HpaBpl-2 1 -----MKPFEDFRADKKRFPPTGCEYLLESLCD-DREIYLYGERVK 37
HpaBpl-3 1 -----MKPENLRITDNKRFPPTGCEYLLESLCD-DREIYLYGERVK 37
HpaBro-1 1 MTTTESAPETALDPTKVNPAADCEANRTANFATRFMTGCEYLLESLCD-DREIYLYGERVK 59
HpaBro-2 1 MTTTEAAPET-VDRTKVNPAADCEANRTANFATRFMTGCEYLLESLCD-DREIYLYGERVK 58
HpaBro-3 1 -----MTTSAFVDAPVAAPNDVRFMTGCEYLLESLCD-DREIYLYGERVK 43

HpaBtt 28 DVTTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 97
HpaBpa 38 DVTTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 97
HpaBec 38 DVTTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 97
HpaBpl-1 45 DVVTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 104
HpaBpl-2 38 DVTTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 97
HpaBpl-3 38 DVTTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 97
HpaBro-1 60 DVVTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 119
HpaBro-2 59 DVVTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 118
HpaBro-3 44 DVVTHPAFRNPAASIGQLYDALHAFETHDILCWNVDTGSGGVTHERFFRFAISADDLRQR 103

HpaBtt 84 GCRYKIMADONLIGMGRSPDYLNAMVVMAYASADYEFEEAENVRNYRYLRDQDEATRA 143
HpaBpa 98 -DAIAEWSRLTYGWMGRFPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 156
HpaBec 98 -DAIAEWSRLTYGWMGRFPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 156
HpaBpl-1 105 -DAIAEWSRLTYGWMGRFPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 163
HpaBpl-2 98 -DAIAEWSRLTYGWMGRFPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 156
HpaBpl-3 98 -DAIAEWSRLTYGWMGRFPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 156
HpaBro-1 120 -DAIAEWSRLTYGWMGRSPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 178
HpaBro-2 119 -DAIAEWSRLTYGWMGRSPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 177
HpaBro-3 104 -DAIAEWSRLTYGWMGRSPDYKAAFGSALGANPGEYGFEDNAKTYKRLQACELLYNHA 162

HpaBtt 144 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 200
HpaBpa 157 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 213
HpaBec 157 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 213
HpaBpl-1 164 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 219
HpaBpl-2 157 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 213
HpaBpl-3 157 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 212
HpaBro-1 179 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 234
HpaBro-2 178 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 233
HpaBro-3 163 IINPPIDRRKLPDEIDVDFIQLF--KETDAGLIIVSGAKVVATNSALTHYNFIC-FGSAQL 218

HpaBtt 201 QAGSEKYALFRLTSTFGLHFVCREA--LVGQ--LSPFFLPLSSRFENDAILLMDKVL 256
HpaBpa 214 LGDNTDFALMFAIPMNTFGKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 273
HpaBec 214 MGENPDFALMFAIPMNTFGKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 273
HpaBpl-1 220 I-KKRFALICTVPMDDAPGVKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 278
HpaBpl-2 214 MGDNPDFALMFAIPMNTFGKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 273
HpaBpl-3 213 IGDNPDFALMFAIPMNTFGKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 272
HpaBro-1 235 I-KKRFALICTVPMDDAPGVKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 293
HpaBro-2 234 I-KKRFALICTVPMDDAPGVKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 292
HpaBro-3 219 L-RKKKEYGLICTVPMDDAPGVKLISSSYELVAGIAGSPFDYPLSSRFENDAILLMDKVL 277

HpaBtt 257 VPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 313
HpaBpa 274 IPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 330
HpaBec 274 IPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 330
HpaBpl-1 279 VPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 335
HpaBpl-2 274 IPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 330
HpaBpl-3 273 IPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 329
HpaBro-1 294 VPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 350
HpaBro-2 293 VPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 349
HpaBro-3 278 VPWENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 333

HpaBtt 314 VY-ENVEFLGMVBLCNNAYAATGALNHMA-HQVVALKTAETAEPLGVAAAM-A-AGTAD 368
HpaBpa 331 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 385
HpaBec 331 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 385
HpaBpl-1 336 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 390
HpaBpl-2 331 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 385
HpaBpl-3 330 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 384
HpaBro-1 351 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 405
HpaBro-2 350 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 404
HpaBro-3 334 EFRG-VQADLGEVWAM-RNL--FWSLTDAMWAEAKPWECSGAYMEDTQ-AICTYHVMABTA 388

HpaBtt 369 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 425
HpaBpa 386 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 445
HpaBec 386 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 445
HpaBpl-1 391 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 450
HpaBpl-2 386 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 445
HpaBpl-3 385 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 444
HpaBro-1 406 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 465
HpaBro-2 405 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 464
HpaBro-3 389 YPRIKETIEEDVPSGLIYLPSARDFKSDVRFYLDKYVRGSGCMFAVERVKIMKALMDS 448

HpaBtt 426 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 481
HpaBpa 446 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 502
HpaBec 446 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 502
HpaBpl-1 451 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 507
HpaBpl-2 446 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 502
HpaBpl-3 445 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 501
HpaBro-1 466 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 522
HpaBro-2 465 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 521
HpaBro-3 449 TISFGARFELYEYEFH--DHYRMYDIT--YVYVYKPEYKERTAFLESKVFEEVQA-- 505

HpaBtt 481 ----- 481
HpaBpa 503 VEDLVGNDVSYFGNK-- 520
HpaBec 503 VEDLVGNDVSYFGNK-- 520
HpaBpl-1 508 GNDVSYFGNK-- 517
HpaBpl-2 503 VEDLVGNDVSYFGNK-- 520
HpaBpl-3 502 VEDLVGNDVSYFGNK-- 519
HpaBro-1 523 VEDLVGNDVSYFGNK-- 538
HpaBro-2 522 VEDLVGNDVSYFGNK-- 537
HpaBro-3 506 VEDLVGNDVSYFGNK-- 520

Fig. S2 Multiple sequence alignment of HpaB enzymes. The HpaB enzymes analyzed are listed in Table S1. HpaB_{tt}, HpaB form *Thermus thermophilus* HB8 (NCBI accession number BAD70783). Asterisks indicate the residues that are involved in substrate binding in HpaB_{tt}.

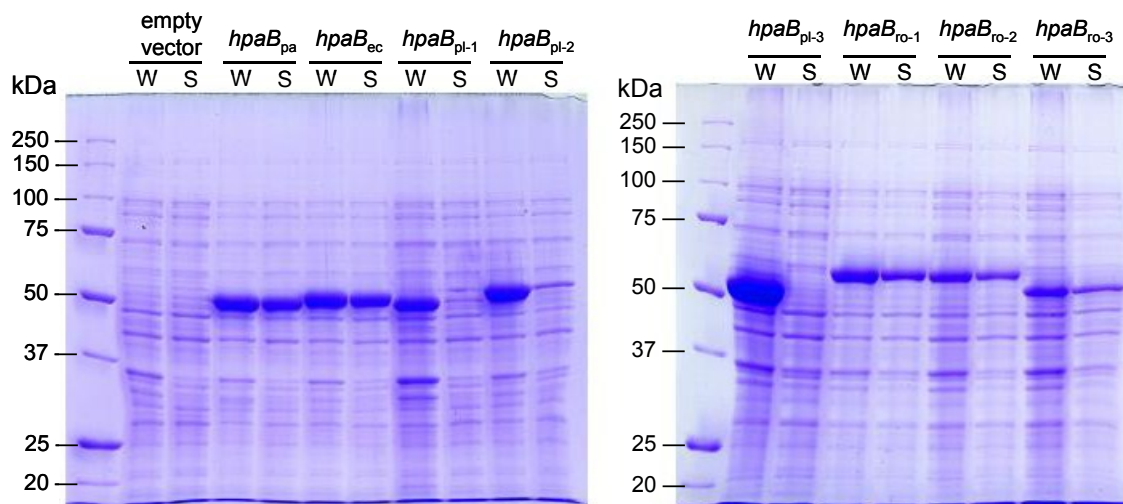


Fig. S3 SDS-PAGE analysis of expression of HpaB enzymes in *E. coli*. Samples prepared from *E. coli* cells carrying the empty vector or respective *hpaB* gene were loaded onto a polyacrylamide gel. W, whole-cell sample; S, soluble-fraction sample.

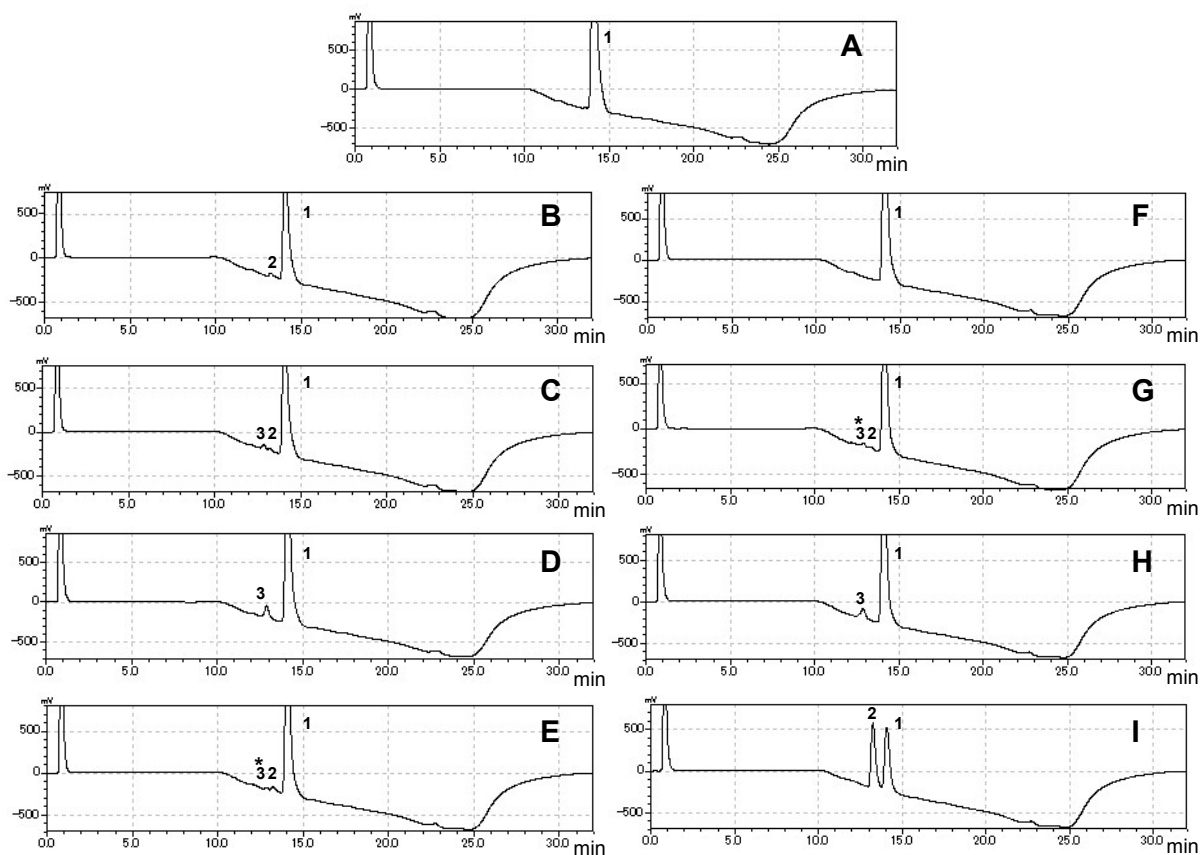


Fig S4 HPLC analysis of reactions of HpaB enzymes with (*S*)-equol. *E. coli* cells carrying the empty vector (A), *hpaB*_{pa} (B), *hpaB*_{ec} (C), *hpaB*_{pl-1} (D), *hpaB*_{pl-2} (E), *hpaB*_{pl-3} (F), *hpaB*_{ro-1} (G), *hpaB*_{ro-2} (H), or *hpaB*_{ro-3} (I) were incubated with (*S*)-equol. Peaks 1 (at 14.0 min), 2 (at 13.2 min), and 3 (at 12.8 min) were found to correspond to (*S*)-equol, (*S*)-3'-hydroxyequol, and (*S*)-6-hydroxyequol, respectively. Peaks marked with asterisks were occasionally not detected.

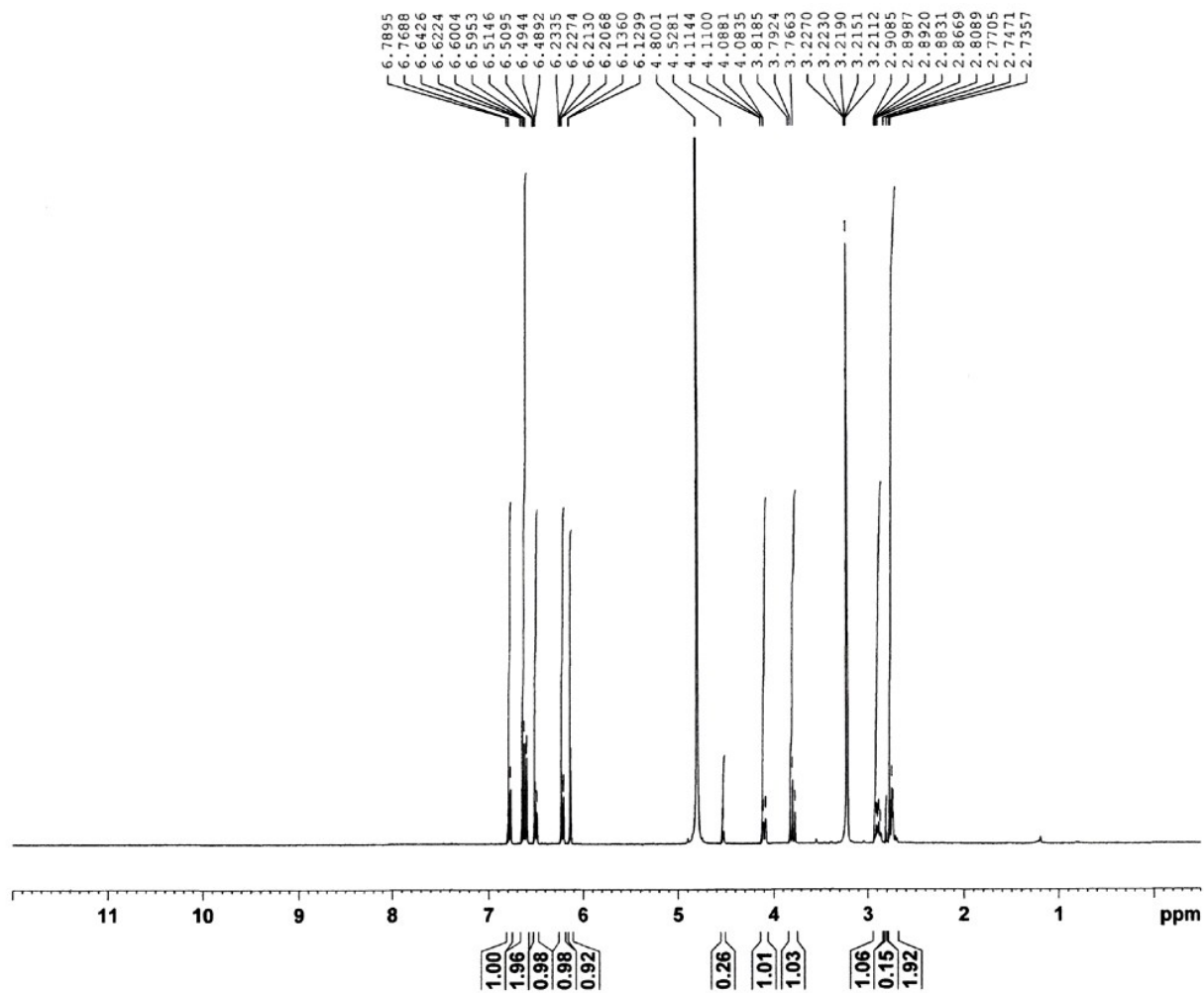


Fig. S5 ^1H NMR spectrum of the reaction product 3'-hydroxyequol.

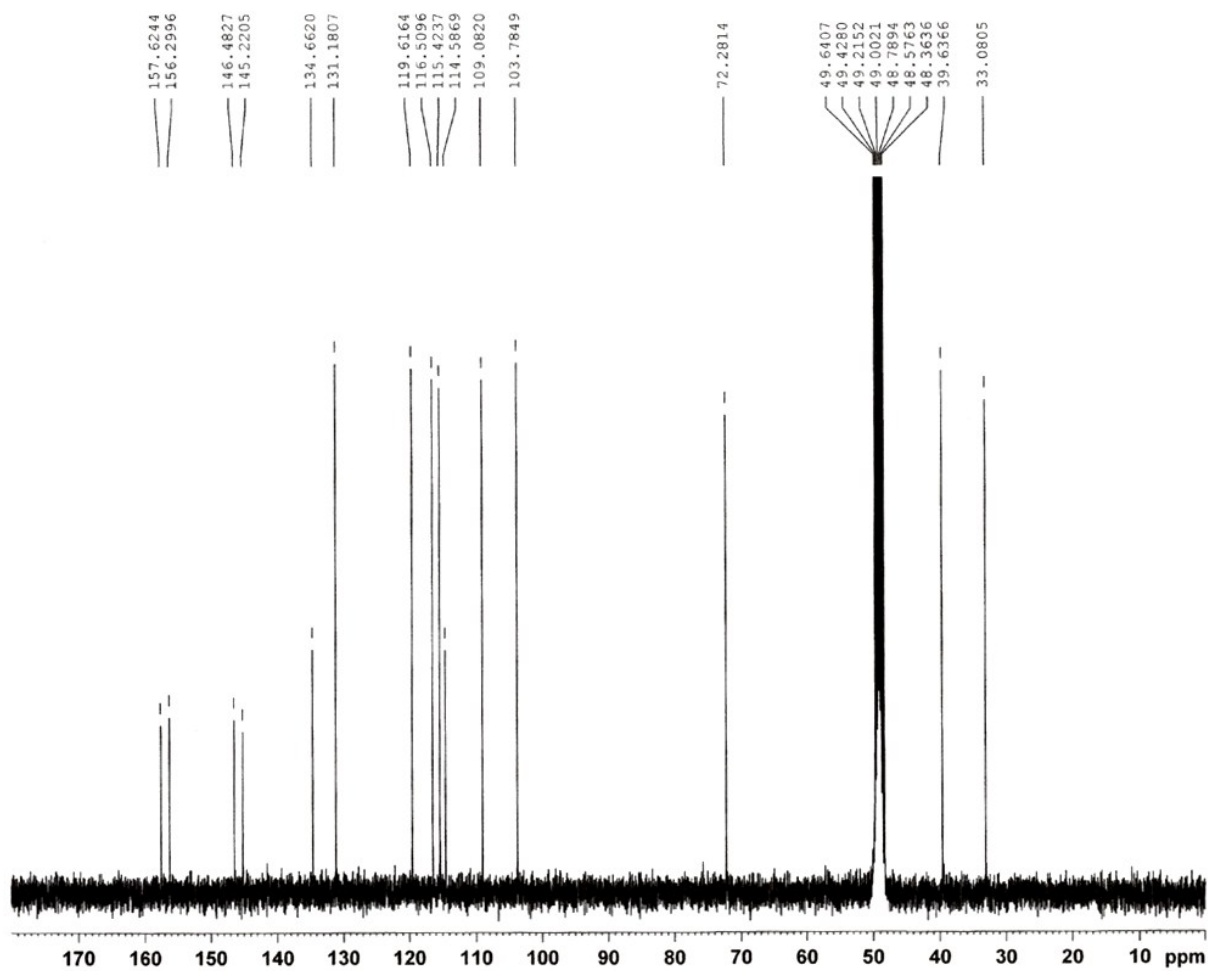


Fig. S6 ¹³C NMR spectrum of the reaction product 3'-hydroxyequol.

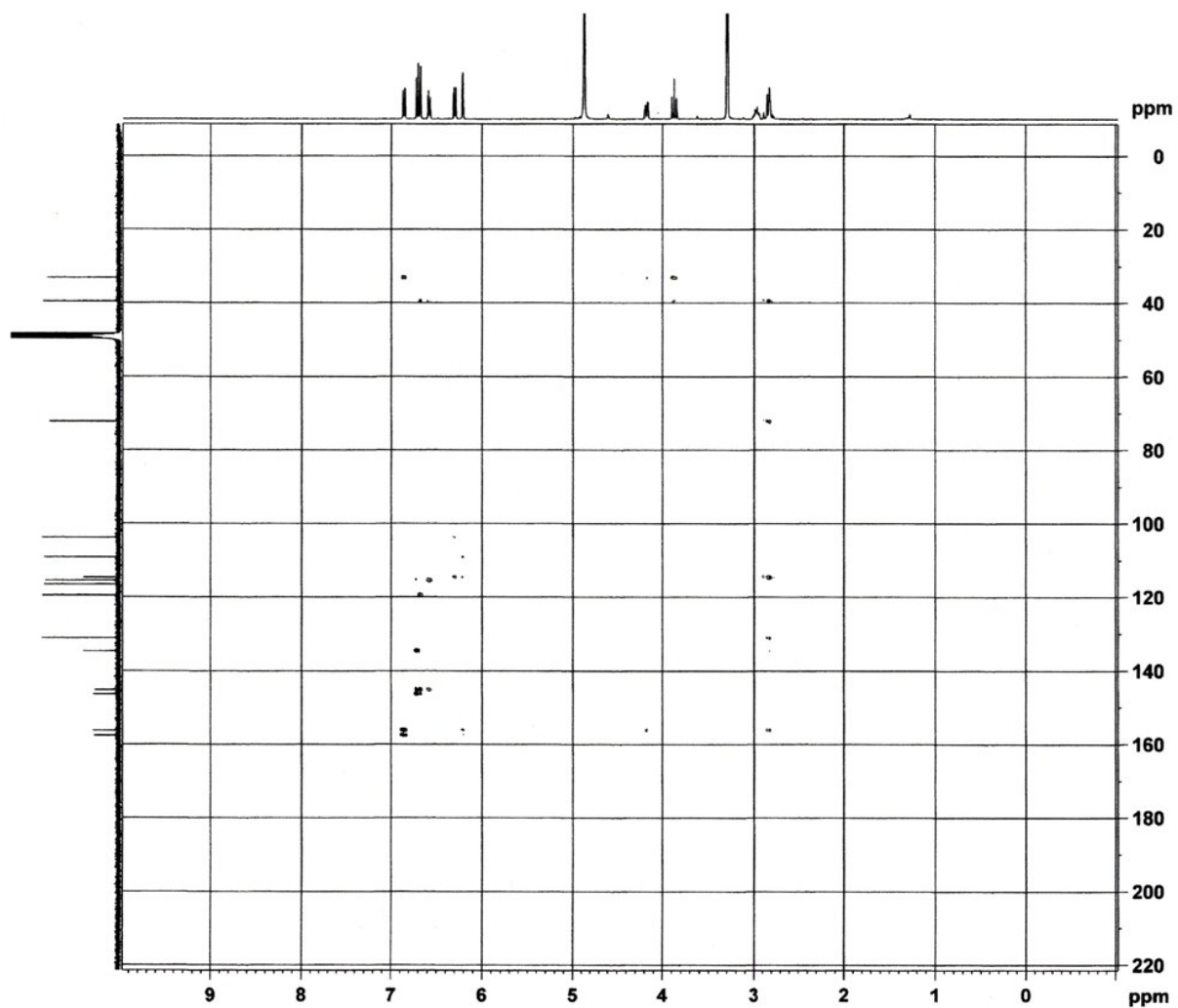


Fig. S7 HMBC spectrum of the reaction product 3'-hydroxyequol.

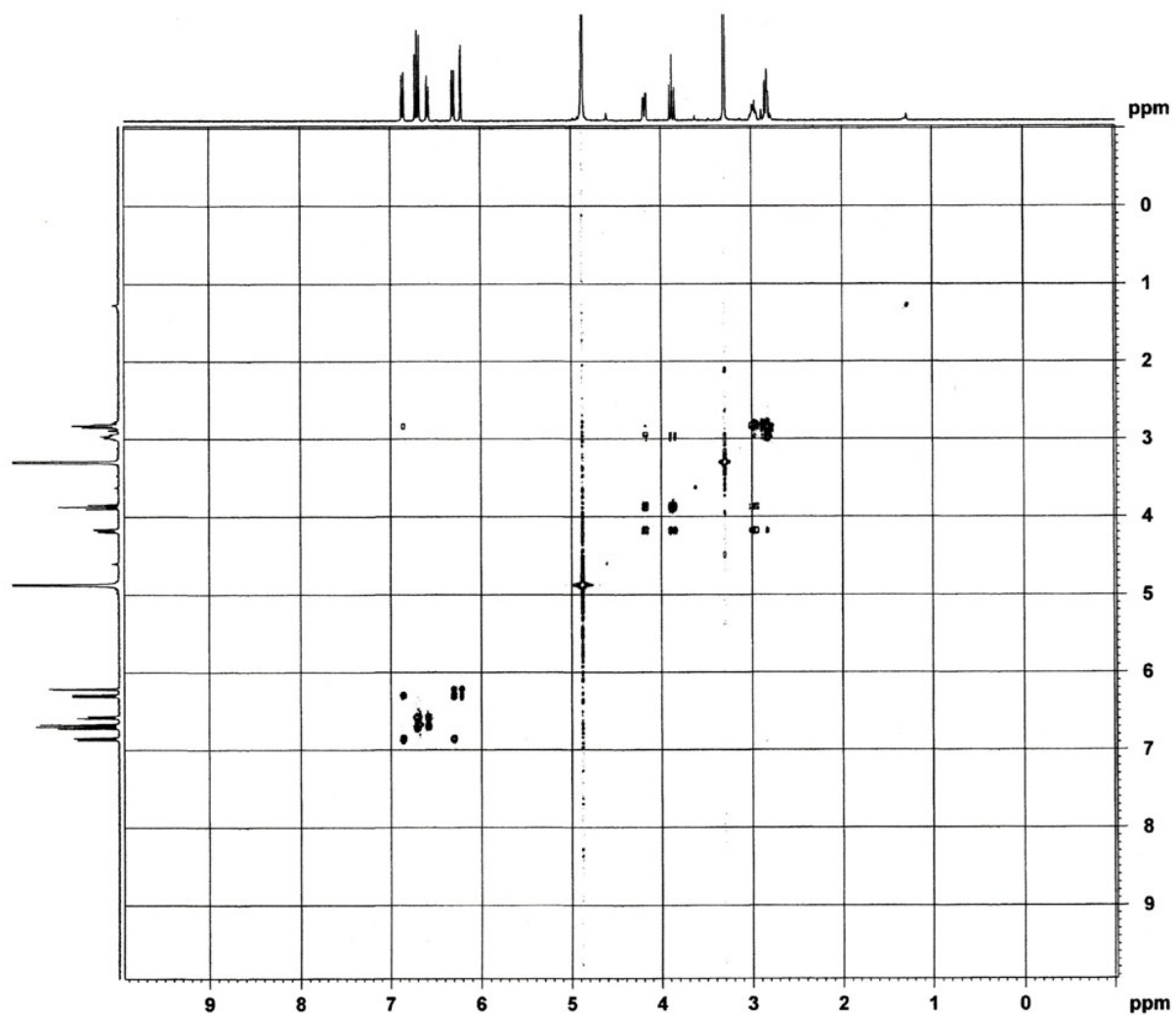


Fig. S8 COSY spectrum of the reaction product 3'-hydroxyequol.

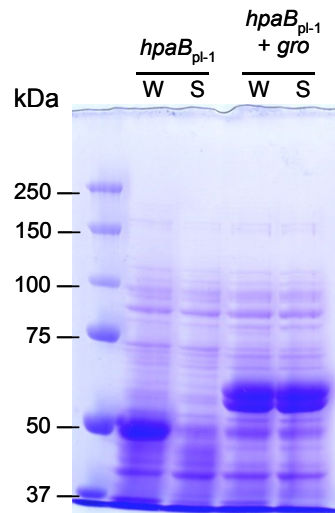


Fig. S9 SDS-PAGE analysis of expression of HpaB_{pl-1} in *E. coli*. Samples prepared from *E. coli* cells carrying $hpaB_{pl-1}$ or $hpaB_{pl-1}$ and pGro7 were loaded onto a polyacrylamide gel. W, whole-cell sample; S, soluble-fraction sample.

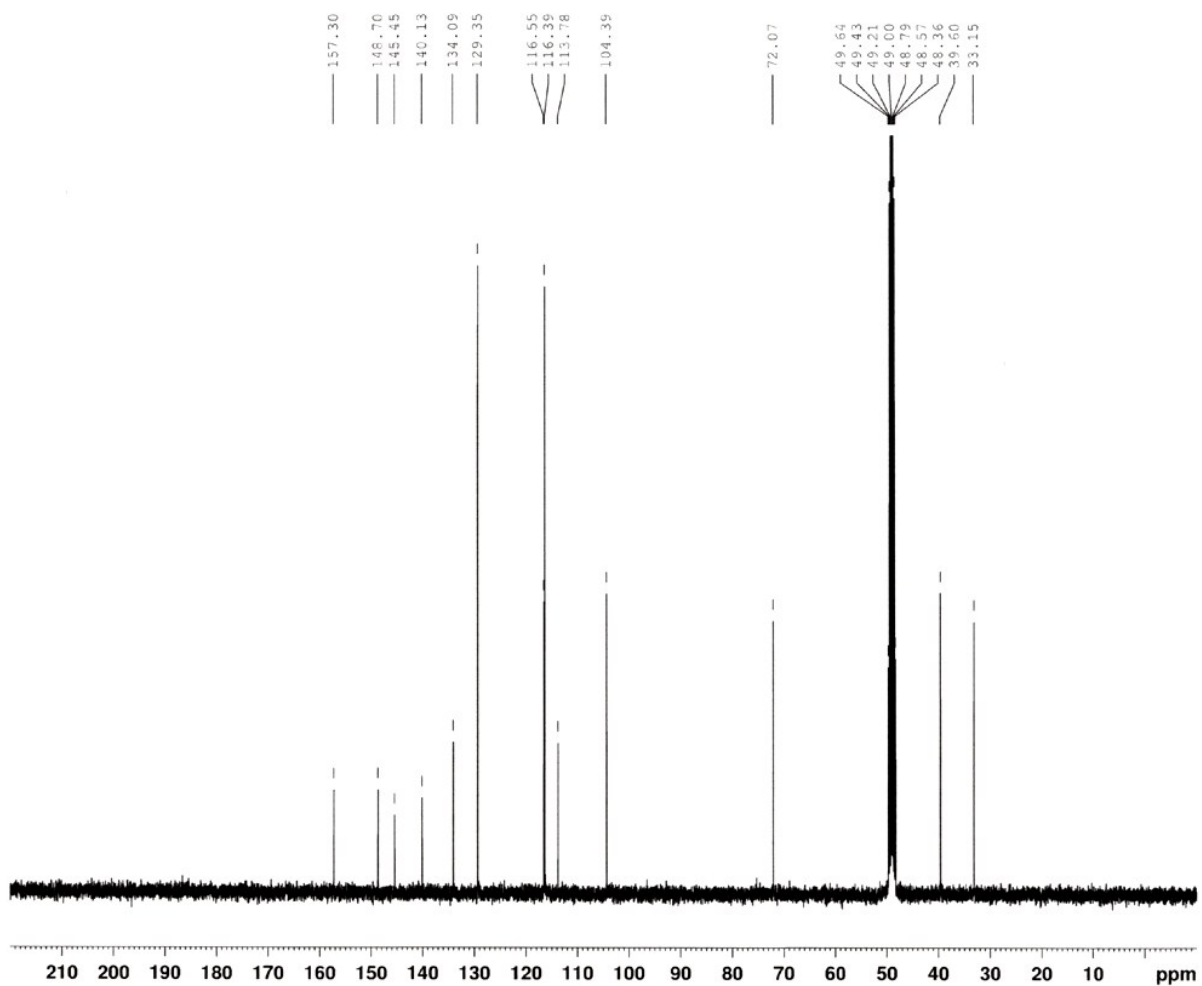


Fig. S11 ^{13}C NMR spectrum of the reaction product 6-hydroxyequeol.

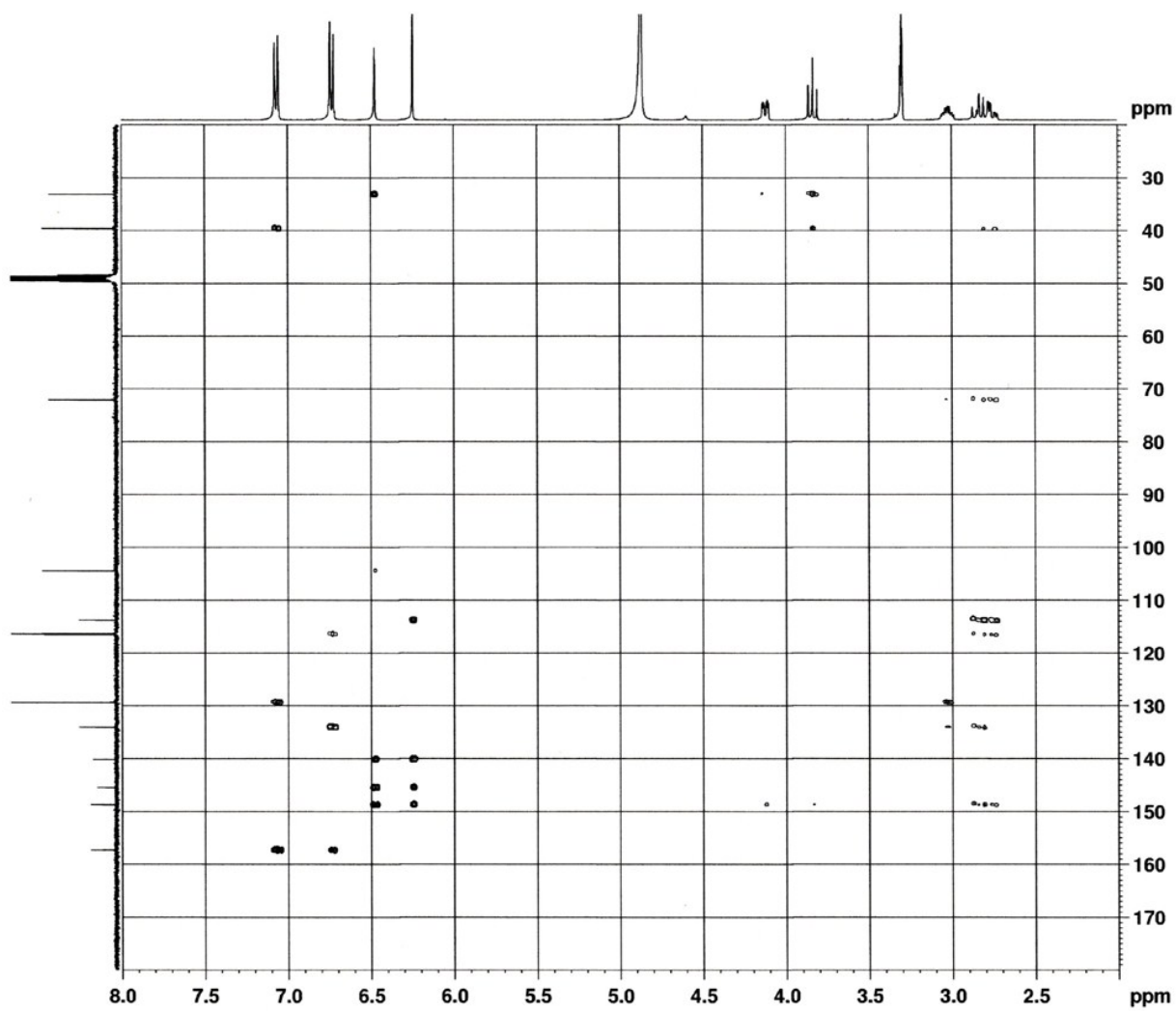


Fig. S12 HMBC spectrum of the reaction product 6-hydroxyequol.

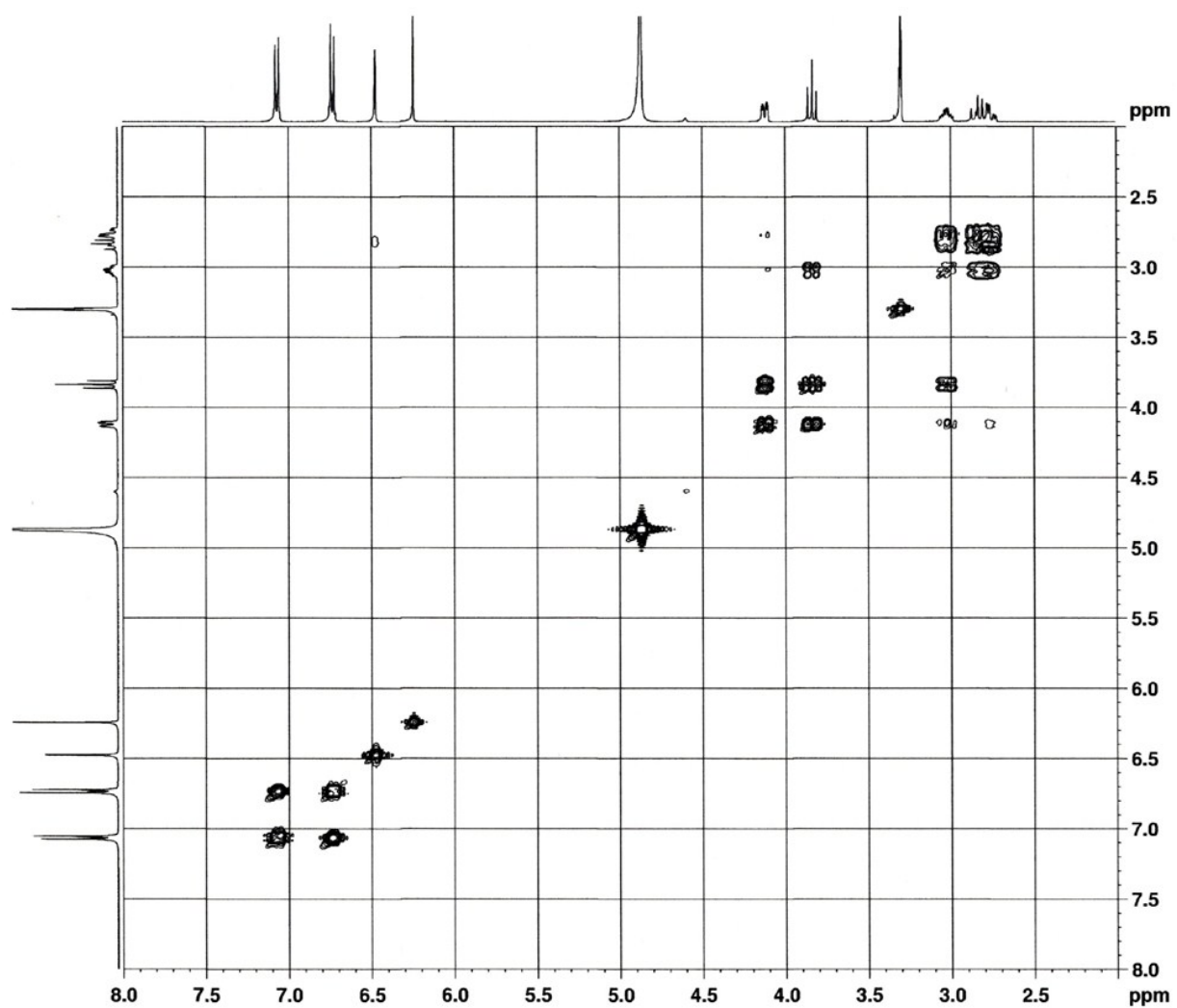


Fig. S13 COSY spectrum of the reaction product 6-hydroxyequol.