Electric supplementary information (ESI)

Monooxygenase-catalyzed regioselective hydroxylation for the synthesis of hydroxyequols

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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, $hpaB_{pl\text{-}1}$, $hpaB_{pl\text{-}2}$, and $hpaB_{pl\text{-}3}$ (Table S1). The genome sequence of *Rhodococcus opacus* B-4 contains three *hpaB* homologues, $hpaB_{ro\text{-}1}$, $hpaB_{ro\text{-}2}$, and $hpaB_{ro\text{-}3}$ (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 (OD_{600}=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 $hpaB_{pl\text{-}1}$ 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 $hpaB_{pl\text{-}1}$, 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 (OD_{600}=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 μL) 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 (4.6×20 mm; particle size, 3.5 μm; Waters, Milford, MA, USA). The reaction mixture (250 μL) was acidified by the addition of HCl (pH 2–3), and methanol (500 μL) and water (250 μL) were then added. The solution was vigorously shaken and centrifuged. The resulting supernatant (10 μL) 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: ^{1}H 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); ^{13}C 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 $C_{15}H_{13}O_4$ $[M-H]^-$: 257.0814, found: 257.0815.

6-Hydroxyequol: ^{1}H 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 $C_{15}H_{13}O_4$ $[M-H]^-$: 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]
hpaB$_{pl-1}$	CAE12541	GAATTCAC**ATG**TCAGAAAACATGAAACAAAAAT (forward)	PciI
		CGCGGATCC**CTA**GCGGTTGGAAGGCCAAAT (reverse)	BamHI
hpaB$_{pl-2}$	CAE13270	TTCTC**ATG**AAACCAGAAGATTTTCGCGCCG (forward)	BspHI
		CGCGGATCC**TTA**TTTCAGCAGCTTATCTAA (reverse)	BamHI
hpaB$_{pl-3}$	CAE16399	TTCTC**ATG**AAACCAGAAAATTTACGTACCG (forward)	BspHI
		CGCGGATCC**TTA**CTTTAGTAATTTATCTAA (reverse)	BamHI
hpaB$_{ro-1}$	BAH50341	TTCCAT**ATG**ACCACCACCGAAAGCGCACCC (forward)	NdeI
		GCGCAATTG**CTA**CTTGTTACCGAAGTACGA (reverse)	MunI
hpaB$_{ro-2}$	BAH50488	TTCCAT**ATG**ACCACCACCGAAGCTGCCCCC (forward)	NdeI
		GCGCAATTG**CTA**GCTGCGGCCGAAGTAGGA (reverse)	MunI
hpaB$_{ro-3}$	BAH51988	TTCCAT**ATG**ACCACATCAGCTTTCGTCGAC (forward)	NdeI
		GCGCAATTG**TCA**GCTCCGGACGATCCGCAG (reverse)	MunI
hpaB$_{ec}$	CAQ34705	TTCTC**ATG**AAACCAGAAGATTTCCGCGCCA (forward)	BspHI
		CGCGGATCC**TTA**TTTCAGCAGCTTATCCAG (reverse)	BamHI
hpaB$_{pa}$	AAG07478	TTCTC**ATG**AAACCCGAAGATTTCCGTGCCT (forward)	BspHI
		CGCGGATCC**TCA**TTGGCGGATGCGATCGAG (reverse)	BamHI
hpaC$_{pa}$	AAG07479	TTCCAT**ATG**TCCCAGCTCGAACCCAGGCAG (forward)	NdeI
		TTCGGTACC**TCA**GGCCGCCCGCCGGGGGCA (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.

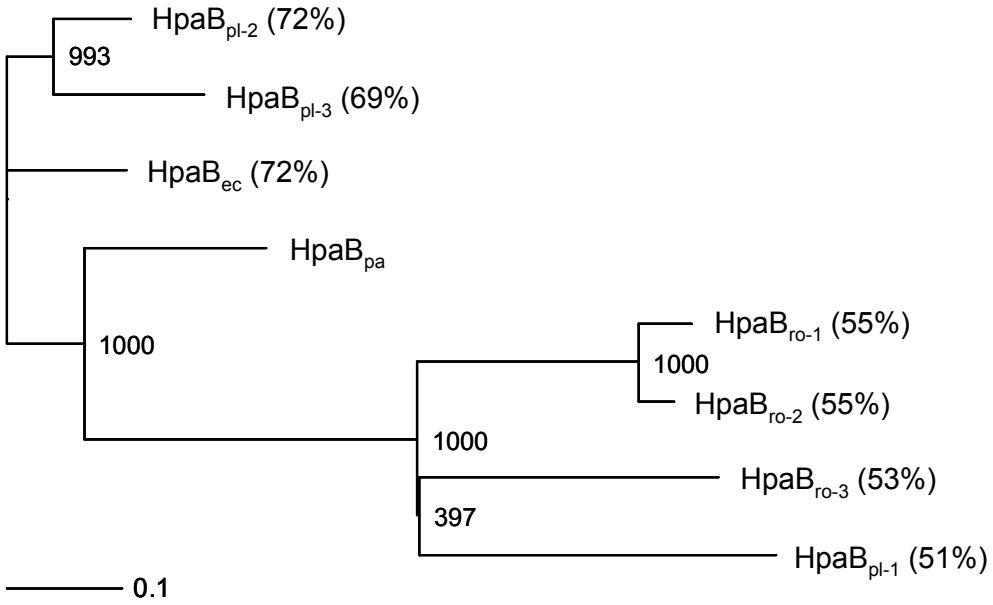

Fig. S1 Phylogenetic tree of eight HpaB enzymes. The HpaB enzymes analyzed are listed in Table S1. Amino acid identity between $HpaB_{pa}$ and other HpaB enzymes are shown in parentheses.

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 $HpaB_{tt}$.

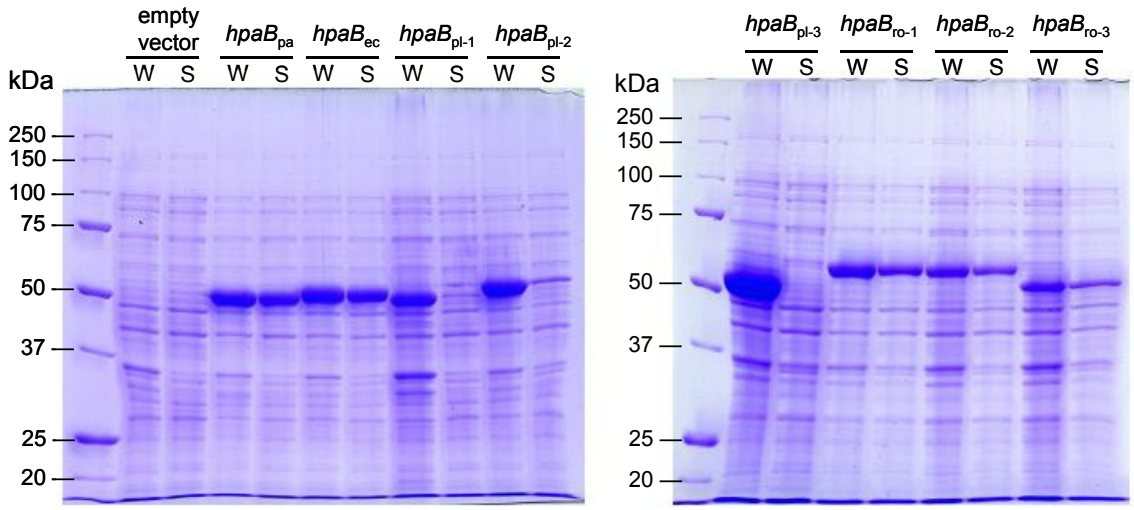

Fig. S3 SDS-PAGE analysis of expression of HpaB enzymes in *E. coli*. Samples prepared from *E. coli* cells carrying the empty vector or respective *hpaB* gene were loaded onto a polyacrylamide gel. W, whole-cell sample; S, soluble-fraction sample.

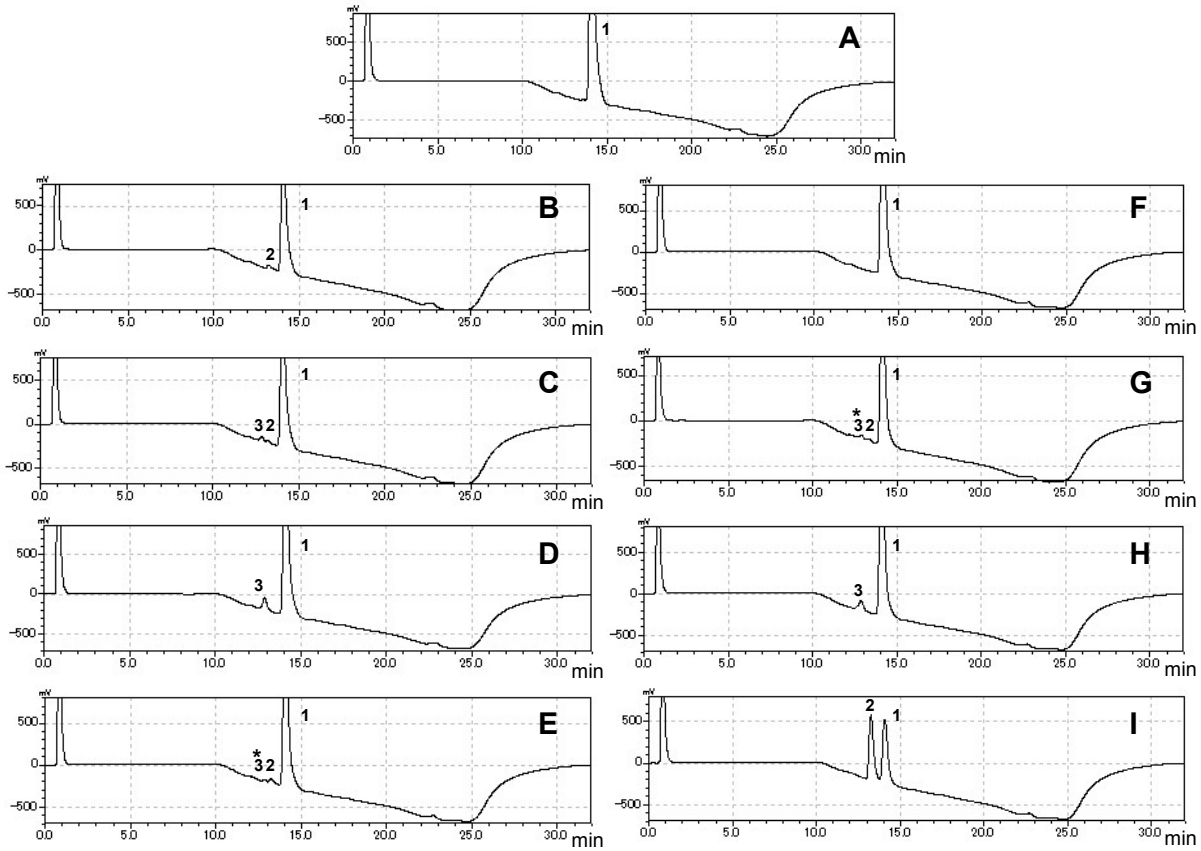

Fig S4 HPLC analysis of reactions of HpaB enzymes with (*S*)-equol. *E. coli* cells carrying the empty vector (A), *hpaB*$_{pa}$ (B), *hpaB*$_{ec}$ (C), *hpaB*$_{pl-1}$ (D), *hpaB*$_{pl-2}$ (E), *hpaB*$_{pl-3}$ (F), *hpaB*$_{ro-1}$ (G), *hpaB*$_{ro-2}$ (H), or *hpaB*$_{ro-3}$ (I) were incubated with (*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.

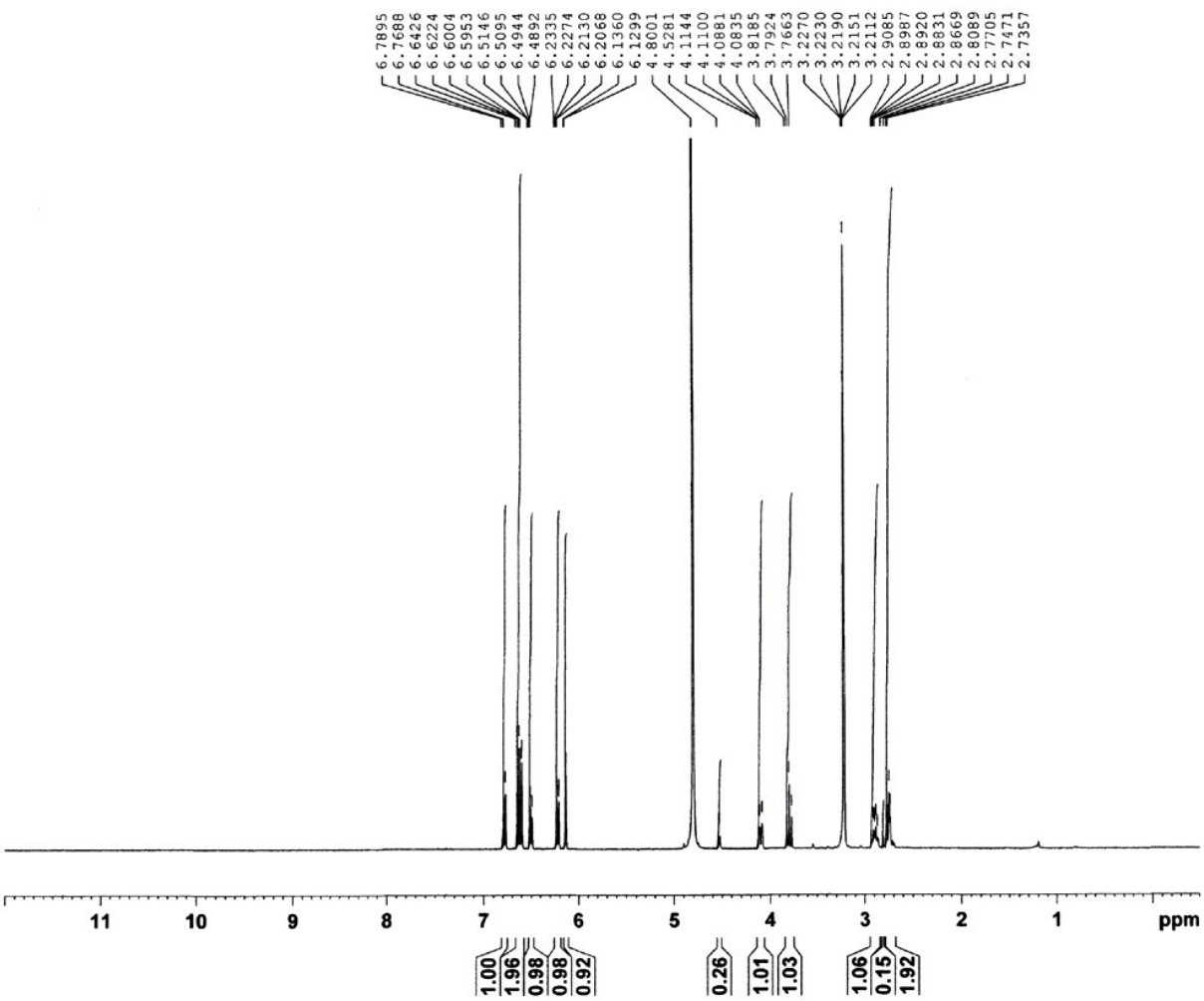

Fig. S5 ^{1}H NMR spectrum of the reaction product 3′-hydroxyequol.

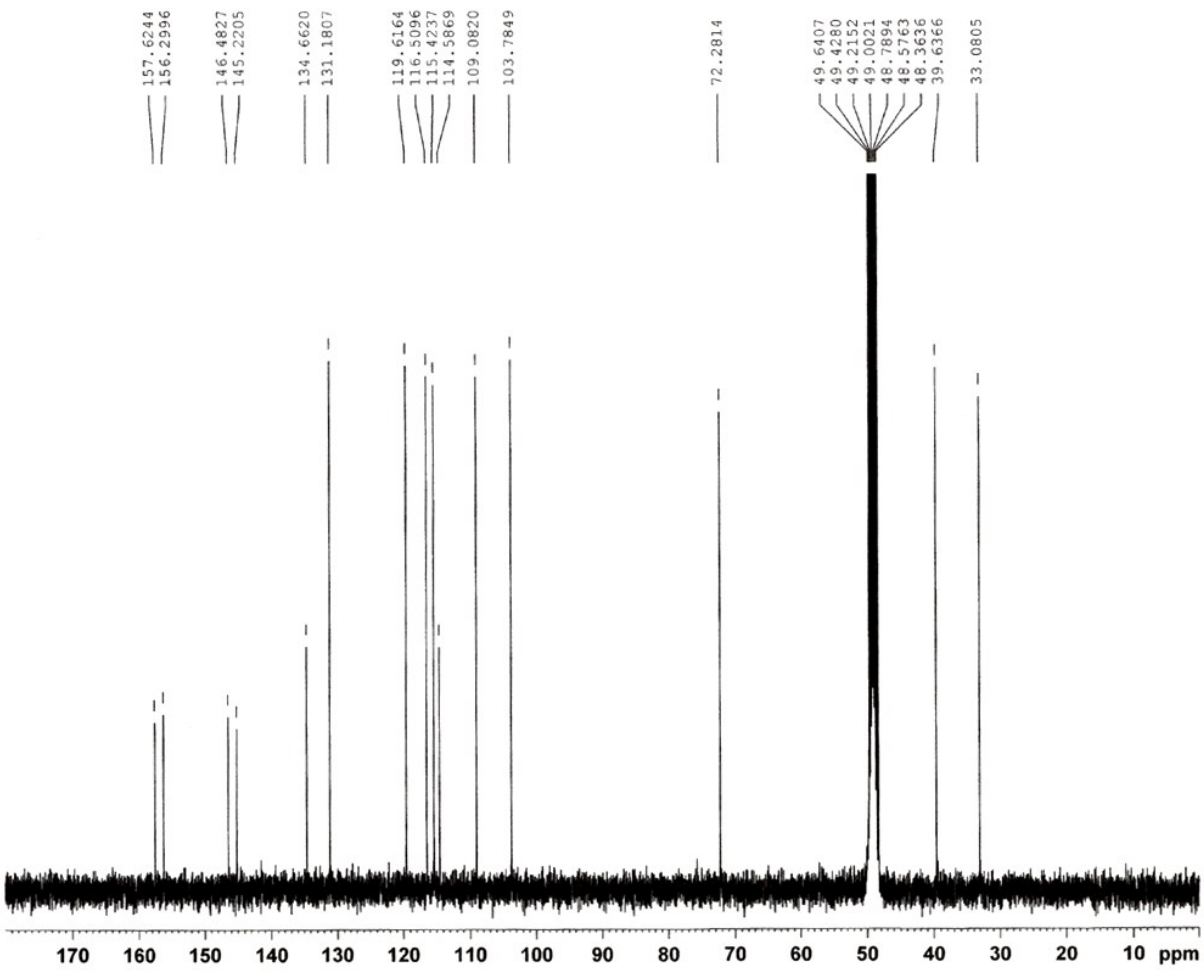

Fig. S6 ^{13}C NMR spectrum of the reaction product 3′-hydroxyequol.

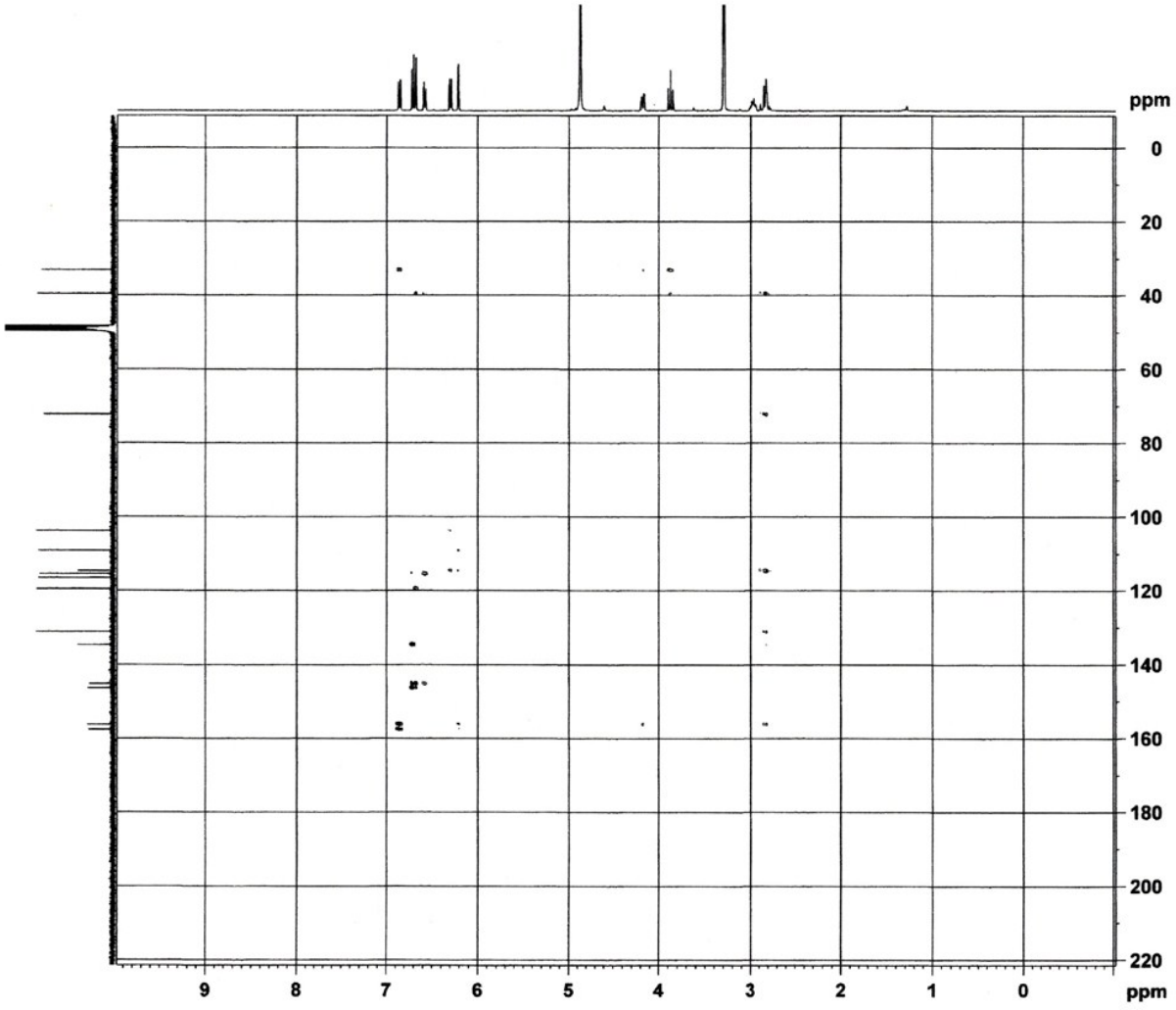

Fig. S7 HMBC spectrum of the reaction product 3′-hydroxyequol.

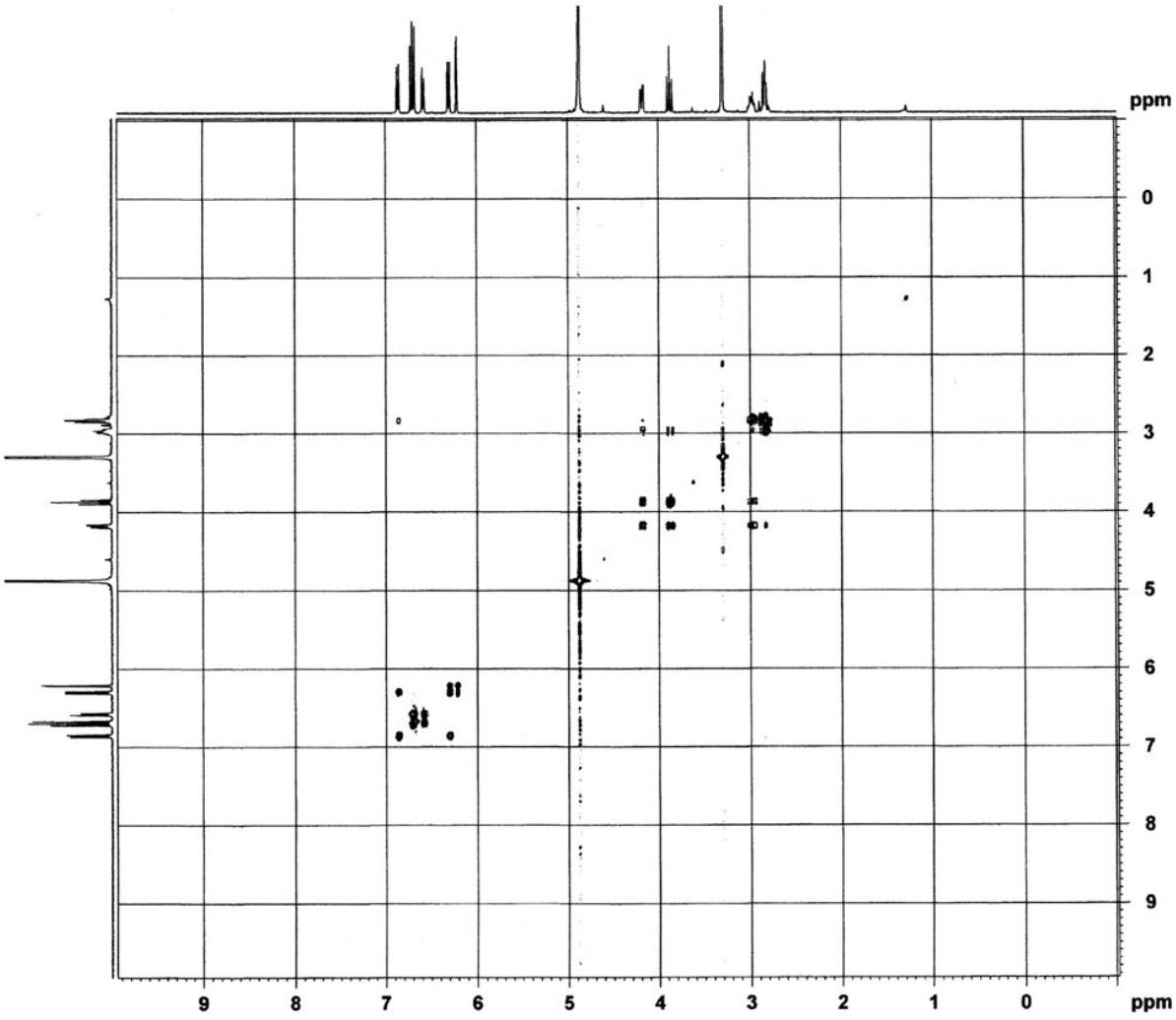

Fig. S8 COSY spectrum of the reaction product 3′-hydroxyequol.

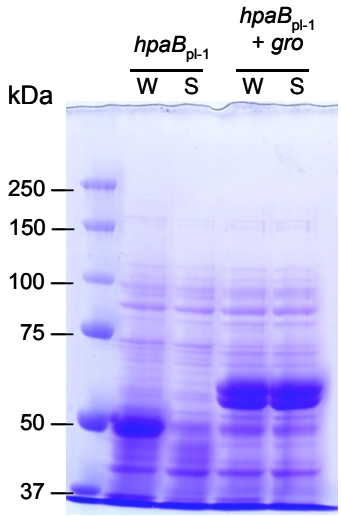

Fig. S9 SDS-PAGE analysis of expression of $HpaB_{pl-1}$ in *E. coli*. Samples prepared from *E. coli* cells carrying *hpaB* $_{pl-1}$ or $hpaB_{pl-1}$ and pGro7 were loaded onto a polyacrylamide gel. W, whole-cell sample; S, soluble-fraction sample.

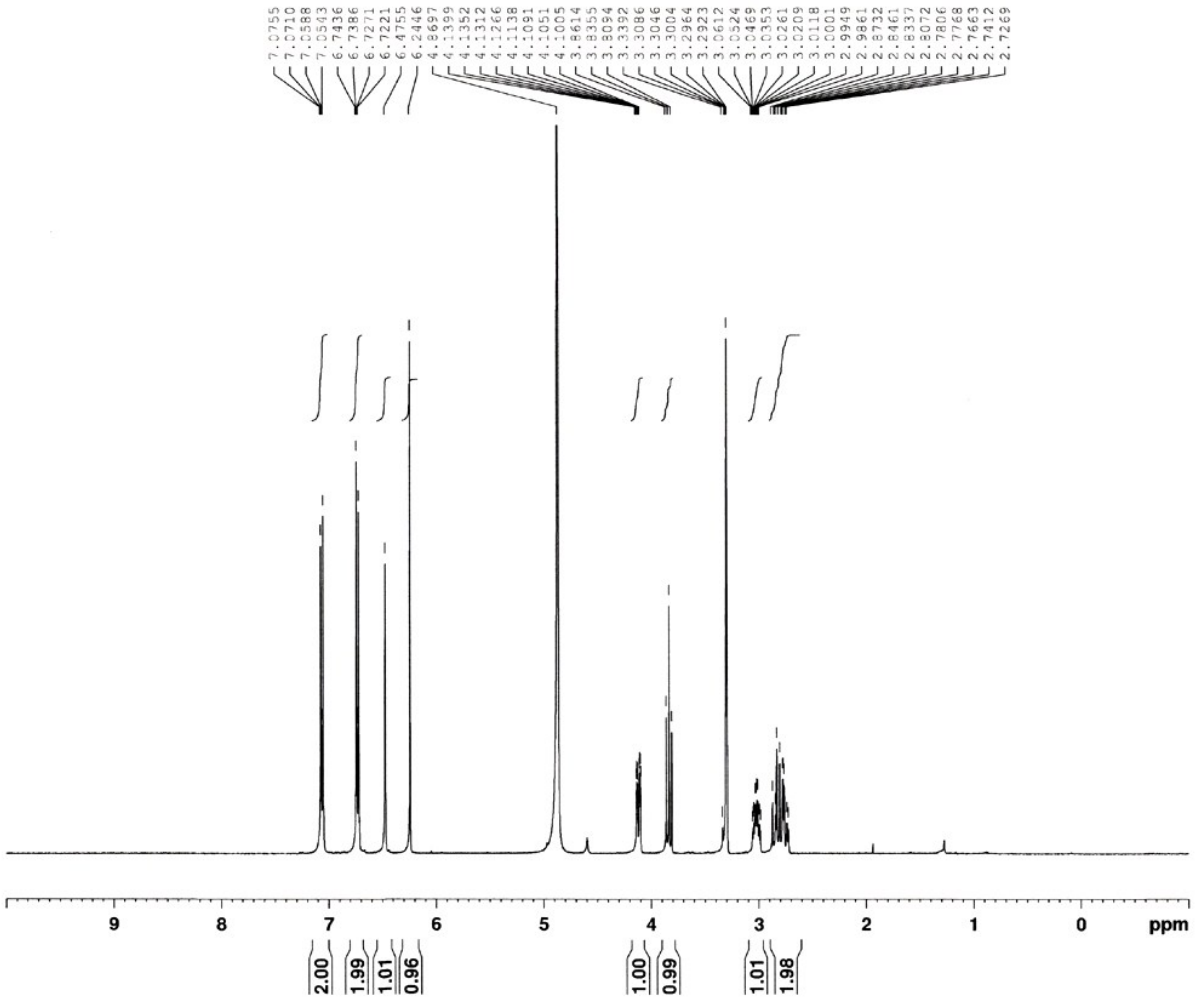

Fig. S10 ^{1}H NMR spectrum of the reaction product 6-hydroxyequol.

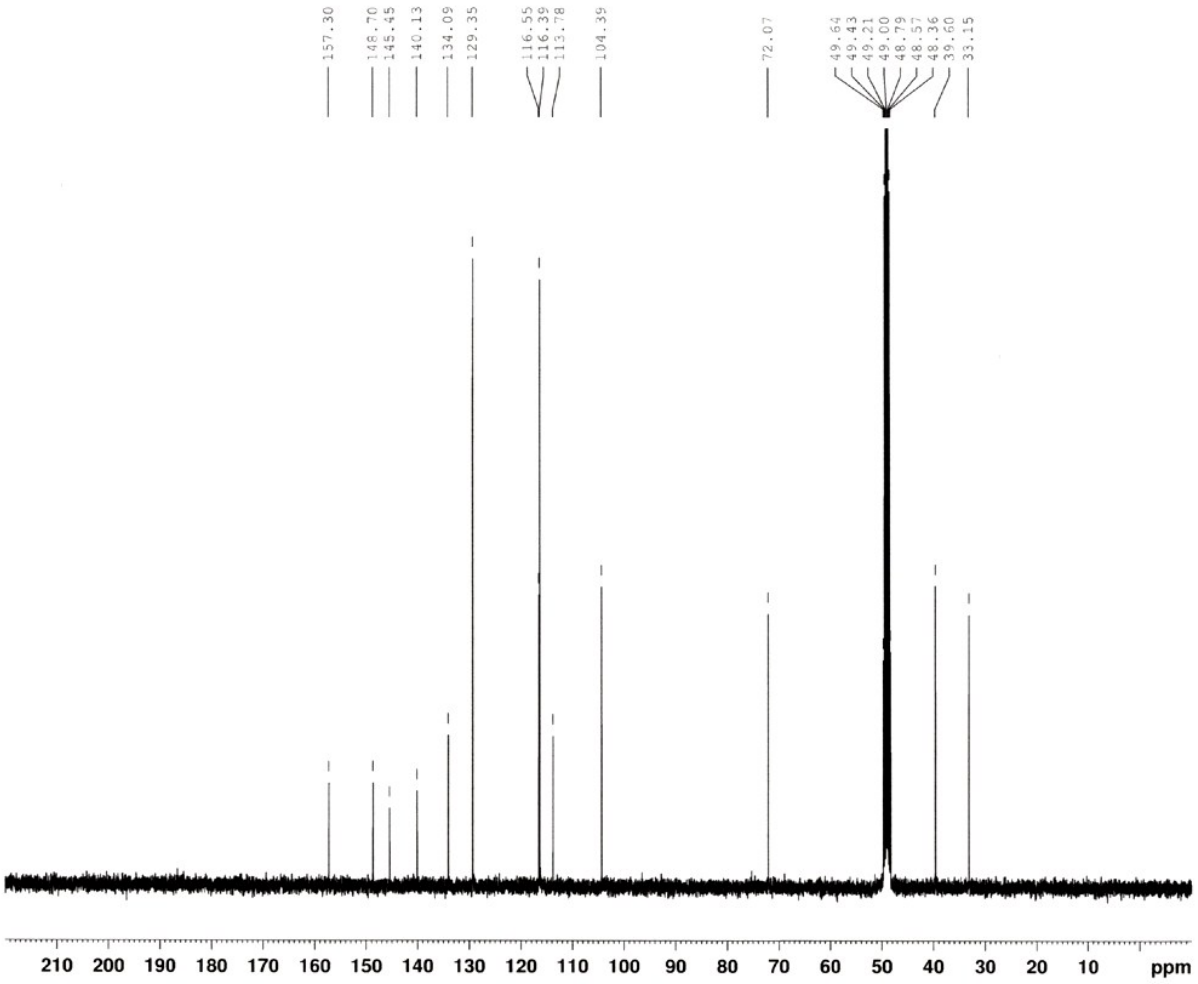

Fig. S11 ^{13}C NMR spectrum of the reaction product 6-hydroxyequol.

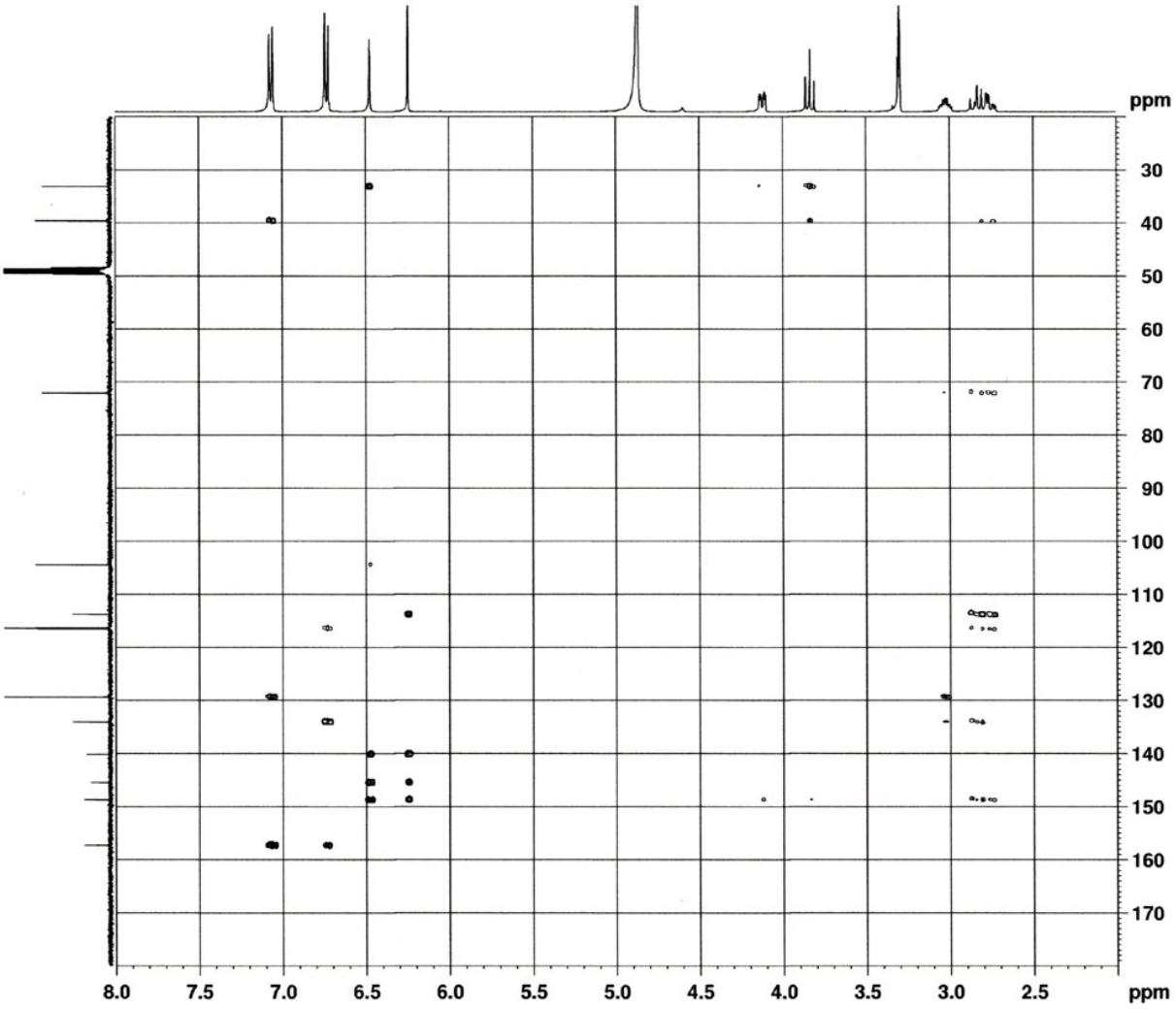

Fig. S12 HMBC spectrum of the reaction product 6-hydroxyequol.

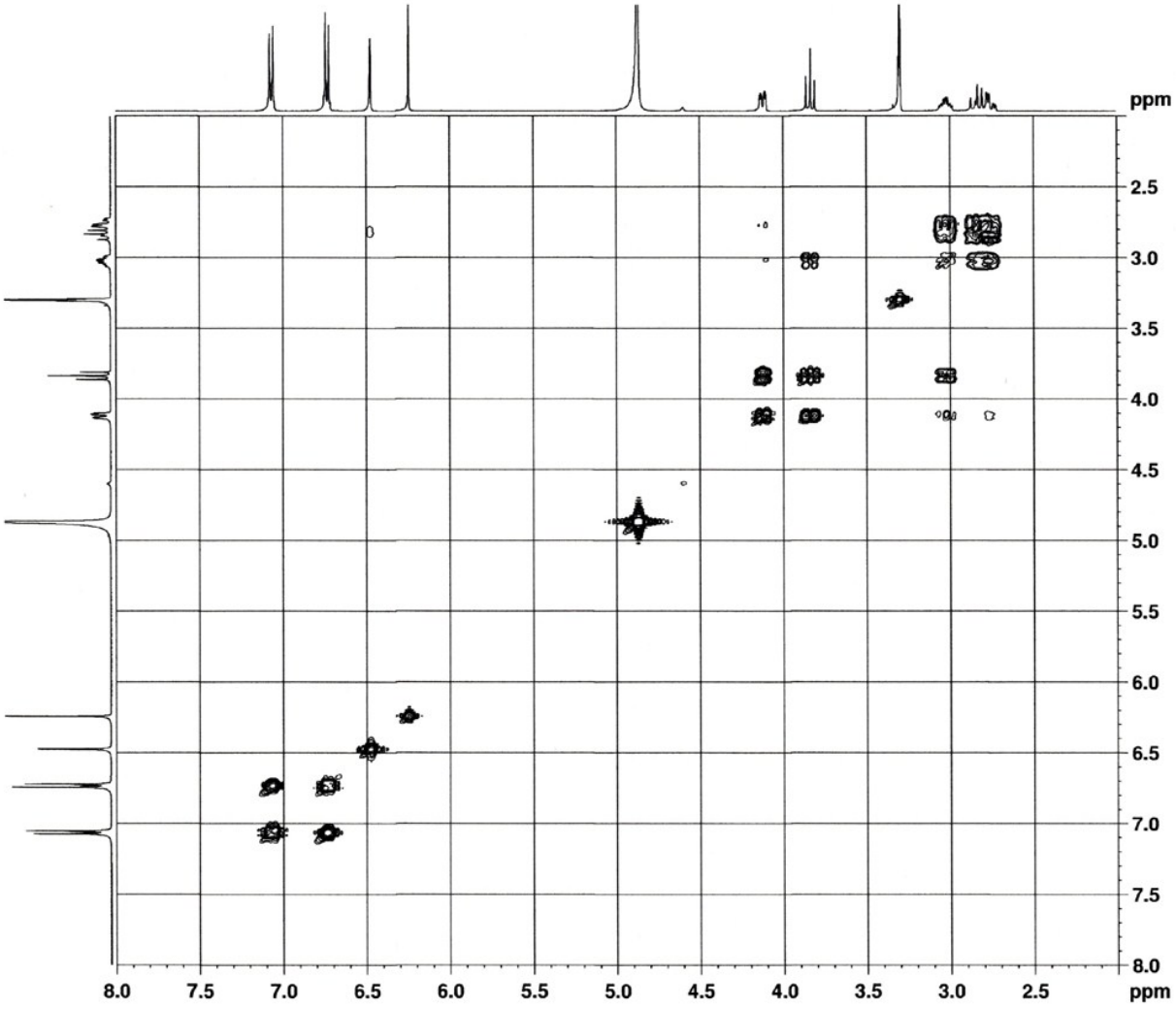

Fig. S13 COSY spectrum of the reaction product 6-hydroxyequol.