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journal or publication title	Biocatalysis and agricultural biotechnology
volume	3
number	4
page range	129-133
year	2014-10
URL	http://hdl.handle.net/10258/3823

doi: info:doi/10.1016/j.bcab.2014.09.005

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Biotransformation of organic compounds *in vivo* using larvae of beetles (*Allomyrina dichotoma*) as biocatalysts

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Abstract

The biotransformation of organic compounds using the larvae of the Japanese rhinoceros beetle (*Allomyrina dichotoma*) as a biocatalyst is described. When phenyl alkanediones were administered by mouth (*p.o*) or subcutaneous injection (*s.c*) to the beetle, asymmetric reduction occurred to yield the corresponding diols in varying optical yields: 1-phenyl-1,2-propandione or 1-phenyl-1,3-butanedione reduced to (1*R*,2*S*)- and (1*S*,2*S*)-1,2-phenylpropanediols in high optical yields or (1*R*,3*S*)- and (1*R*,3*R*)-1-phenyl-1,3-butanediols in low to high optical yields, respectively. By administrating 1-phenyl-1-propanone, 1-phenyl-1-butanone or 4-phenyl-2-butanone, redox reactions occurred to give 1-phenyl-1,2-propanediols or 1-phenyl-1,3-butanediols in lower optical yields. The administrations of β-ionone and cinnamyl chloride resulted in regioselective allylic oxidations producing enone and cinnamic acid, respectively. However, when (*R*)-(-)-carvone was administered, regiospecific dihydroxylation at the isopropenyl group occurred to give (4*R*,8*R*)- and (4*R*,8*S*)-8,9-dihydroxy-8,9-dihydrocarvone as diastereoisomers. These results appear to demonstrate similar reaction tendency with the case of a microorganism. It is possible that these reactions were due in part to bacteria in the intestine of the larva: however, regio- and stereoselectivities of the reactions were sometimes unique. Thus, it is supposed that these biotransformations were accomplished by the ensemble of the larva's own enzymes with several bacteria. The results obtained in this study might show the possibility of using such enzymes derived from insects, including beetle larvae, as a biocatalyst.

Highlights

- We examine the biotransformations using larvae of beetle as a new biocatalyst.
- At the phenyalkanones, stereo- and regioselective redox reactions occurred.
- Allylic carbon atom at β-Ionone and cinnamyl choride were oxidized.
- Dihydroxidation at the isopropenyl group of carvone was observed.
- Larvae of the beetle shows substrate specific reactions.

Keywords: beetle larvae, biotransformation, allylic oxidation, biocatalyst, regiospecific dihydroxylation

1. Introduction

Investigations of biotransformation using a biological catalyst have been reported in many laboratories (Drauz et al., 2012). Among them, biotransformation using cutworm (*Spodoplera litura*) larvae, a lepidopterous insect, has been reported recently (Miyazawa and Miyamoto, 2005). In these reports, *in vivo* enzymatic reactions proceeded singularly and substrate-specifically. Thus, the larvae of other insects are expected to also demonstrate unknown transformations of organic compounds. However, further studies of the biotransformation of organic compounds using other insects are scarce. Thus, we report the efficacy of *in vivo* enzymatic reactions in other insect larvae.

We have investigated the *in vivo* biotransformation of organic compounds using the larvae of the Japanese rhinoceros beetle (*Allomyrina dichotoma*). To protect themselves, the larvae of these beetles secrete an antibacterial peptide, defensin, (Miyanoshita et al.,1996) in response to bacterial invasions. We were very interested in their diet, e.g.. *Fagales* litter, such as sawtooth oak (*Quercus acutissima*) (Kiem et al., 2004), which include terpenoids. Thus, based on their diet, we have considered that they would possess a particular enzyme required to metabolize terpenoids. To the best of our knowledge,

there has been no report concerning enzymatic biotransformation using living beetle larvae as a biocatalyst. This study is the first to report that the larvae of beetles can be used successfully as a biocatalyst for the biotransformation of organic compounds.

2. Material and Methods

2.1 Instruments

All reactions were conducted in oven-dried glassware. Silica gel 60 (70-230 mesh, ASTM, Merck) was used for column chromatography (eluent:n-hexane or n-hexane:chloroform =1:1 *v*/*v*). Precoated Kieselgel 60F-254 plates (0.25 mm, Merck) were used for TLC analysis. Spots were detected by absorption of UV light at 254 nm. Optical rotations were measured using JASCO DIP-360 polarimeters. ^{1}H- and ^{13}C-NMR spectra were recorded using JEOL JNM 600 (600 MHz) spectrometers. Mass spectra were recorded on JEOL JMS DX-303/JMA-DA 5000 spectrometers. The enantiomeric excess (ee) and absolute configurations of the products were determined by HPLC using a Waters 510 HPLC Pump (Waters, Milford MA, USA) equipped with a chiral column (Daicel CHIRALCEL OD-H, AD-H, OB-H) eluted with *n*-hexane:2-propanol at 0.5 mL/min. The eluent was monitored at 254 nm using a Waters 486 Tunable Absorbance Detector (Waters, Milford MA, USA). Chromatographs were recorded using a Chromatocorder 21 (S.I.C., Tokyo, Japan).

2.2 Beetle larvae

The larvae (*Allomyrina dichotoma*: last instar) were purchased from Fine beetle (Minamiuwa, Ehime, Japan). Prior to use, the larvae were reared in cages filled with humus. The total weight of the beetles, which were used for one group, was approximately 800-900 g (average weight ~ 22 g/larva).

2.3 General procedures for administration of chemicals

Liquid chemicals were dissolved in 0.5% Tween 80 and administered by subcutaneous injection (*s.c.*), while solid chemicals were suspended in corn oil and administered by mouth (*p.o.*). Four to six mg of chemicals was administered to each larva once per day for 3 days. A total of 12-18 mg of chemicals was administered to each larva. During this procedure, the larvae were kept in a dark box without soil at approximately 22^{o}C.

2.4 Extractions, isolation and identification of the metabolite

After chopping the larvae with a blender, internal organs and the collected frass were extracted with chloroform using a Soxhlet apparatus. The organic layer was concentrated *in vacuo*, and the residue was subjected to silica gel open-column chromatography. The structure, absolute configurations, and optical yields of the isolated metabolites were identified by comparison with reported data (IR, NMR, MS, and HPLC) using the equipment described in Section 2.1. References for the compounds are cited upon mention in Section 3.

3. Results and discussion

In our initial experiments, we used phenyl alkanone derivatives as substrates for the *in vivo* biotransformation by the beetle larvae. Initially, we selected 1-phenyl-1,2-propanedione (**1**) as a model substrate to determine ideal methods (*p.o.* or *s.c.*). A solution of 1-phenyl-1,2-propanedione (**1**) dissolved in 0.5% Tween 80, was administered to the beetle larvae by *p.o.* and *s.c.* Both administrations yielded two diols, (1*R*,2*S*)- and (1*S*,2*S*)-propanediols (**2** and **3**) (Jiao et al., 2009; Takeshita et al., 1992, 1996; Takeshita and Sato, 1996) at a ratio of 16:1. The chemical and optical yields of diols (**2**) and (**3**) obtained by *p.o.* were 32% and 92%ee, and 2% and 92%ee, respectively. From *s.c.*, the yields were 30% and >97%ee, and 2% and 87%ee, respectively (Table 1). We found no appreciable differences in the biocatalytic activity between *p.o.* and s.c. The results suggest kinetic differences and stereoselectivity of the reduction of 1,2-diketone; the reduction of ketone at the C2-position occurred first to yield 2*S*-alcohol followed by diastereoselective reduction at the C1-position.

(Table 1 around here)

In the case of the administration of 1-phenylpropiophenone (**4**) by *s.c.*, the reduction of

the ketone and regioselective oxidation yielded (*R*)-1-phenyl-1-propanol (**5;** 58% and 34%ee) (Takeshita et al., 1996) and (1*R*,2*R*)-1-phenyl-1,2-propanediol (**6;** 20% and 7%ee) (Jiao et al., 2009; Takeshita et al., 1992), respectively (Scheme 1). The absolute configuration of diol (**6**), in comparison to diols (**2** and **3**) obtained from (**1**), was different and the optical yield was lower. In consideration of this result together with that of compound **1**, this reaction proceeded as follows: The reduction of the ketone at C-1 was followed by diastereoselective oxidation at the C-2 methylene. We also attempted the biotransformation of 1-phenylpropane; however, the substrate was simply recovered in the original amounts.

(Scheme 1 around here)

By administering an emulsion of 1-phenyl-1,3-butanedione (**7**) in corn oil by *p.o*., two diols, (1*R*,3*S*)- and (1*R*,3*R*)-1-pheny-1,3-butanediol (**8** and **9**), were obtained in 11% (87%ee) and 23% (6%ee) yields, respectively (Scheme 2a) (Chorpade et al., 2004; Takeshita et al., 1993, 1995). In this connection, when an emulsion of 4-phenyl-2-butanone (**10**) in corn oil was administered by *s.c*., hydroxylation at the C-4' position of the benzene ring occurred to yield raspberry ketone (**11**) (Beekwilder et al. (2007) in 6% yield, and a redox reaction afforded (2*S*)-4-phenyl-2-butanol (**12**) (Ahmad et al., 2004; Frolander and Moberg, 2009; Kawanami et al., 2009) and diol (**9**), which were isolated with 12% (37%ee) and 5% (24%ee) yields, respectively (Scheme 2b). In addition, by administering an emulsion of butyrophenone (**13**) by *s.c*., three products, (*R*)-1-phenyl-1-butanol (**14**) (Ahmad et al., 2004), (1*S*,3*S*)-1-pheny-1,3-butanediol (**15**), and diol (**8**), were obtained in 19% (53%ee), 22% (69%ee) and 11% (20%ee) yields, respectively (Scheme 2c). We also attempted the biotransformation of 1-phenylbutane; however, the compound was simply recovered in the original amounts.

(Scheme 2 around here)

In the case of the administration of 4-phenyl-3-butyn-2-one (**16**) by *s.c.*, although we expected the triple bond to be reduced to a double or single bond, only the reduction of the ketone occurred to give 4-phenyl-3-butyn-2-ol (**17**) in low optical and chemical yields (34%, 24%ee)(Takeshita et al., 1998) (Scheme 3).

(Scheme 3 around here)

Summarizing the reduction of the ketone and the hydroxylation at the α- or β-hydrocarbons of the ketone, the stereoselectivity of the reduction of the ketone seems to occur only if the oxy-functional group exists near the ketone (**1** and **2**). Otherwise, the ketone was reduced in low stereoselectivity (**4**, **10, 13** and **16**). Hydroxylation of the hydrocarbons occurred if the oxy-functional group exist in that region, and the stereoselectivities were typically very low (showing very moderate diastereoselectivity; **4**, **10** and **13**).

Remarkable substrate-specific reactions were observed as a result of the administration of α–, β–, ĄĄĄ γ-acetylpyridines (**18a**, **b**, **c**) by *s.c*. In the reductions of the acetyl group, the enzymatic affinity for each isomer was very different. The enantioselectivity for the reduction was the highest for α–acetylpyridine (**18a**), followed in order by β–acetylpyridine (**18b**) and γ-acetylpyridine (**18c**) to give α–, β–, and γ–pyridylethanols (**19a**, **b**, **c**) in 15% (60%ee), 12% (18%ee), and 5% (racemic) yields, respectively (Takeshita et al., 1987; Takeshita and Yoshida, 1990; Uwai et al., 2005) (Scheme 4). The stereoselectivities of the reduction of these acetylpyridine derivatives differed form reported data obtained using baker's yeast (Takeshita et al., 1987) and rat liver S-9 fraction (Uwai et al., 2005). Thus, the beetle larvae may be a very unique biocatalyst.

(Scheme 4 around here)

Upon the administration of β-ionone (**20**) to the beetle larvae by *s.c.*, a regioselective redox reaction occurred. As a result, the enone was reduced to saturated alcohol, and the allylic carbon on the cyclohexene ring was oxidized to enone, giving racemic 9-hydroxymegastigm-5-en-4-enone (**21**) (20% chemical yields), which is a natural product from the leaves of *Chamaecyparis formosensis* or *Tectona grandis* (Lin et al., 1999; Macias et al., 2008) (Scheme 5a). This regioselective oxidation at the allylic carbon atom seems to be very similar to the biotransformation of terpenoids with bacteria, yeasts, and fungi (Lupien et al., 1999; Scarder, 2007). However, simultaneous oxidation and reduction in the same compound is very rare. Furthermore, cinnamyl chloride (**22**) was administered to give cinnamic acid (**23**) and the double bond reduced product, propanoic acid **(24**), at a ratio of 14:1 (Scheme 5b). This reaction was also the product of allylic oxidation and successive hydrolysis.

(Scheme 5 around here)

Upon the administration of (*R*)-(-)-carvone (**25**) to the beetle larvae by *s.c.*, two diols, (5*R*,8*R*)- and (5*R*,8*S*)-8,9-dihydroxy-8,9-dihydro-carvone (**26**, **27**) (1:1), were obtained as diastereomeric mixtures (Scheme 6a). In this biotransformation, it is worth noting that regioselective dihydroxylation occurred at the double bond of the isopropenyl group, and the expected allylic oxidation in the cyclohexenone ring (C-4) did not occur. This type of reaction appears to be similar to microbial terpene dihydroxylations (Bicas et al., 2009; Borges et al., 2009). In these reports, the terpene oxidation resulted from sequential epoxidation and spontaneous or enzymatic hydrolysis.

It was presumed that during this reaction, epoxidation of the double bond (C-8) and spontaneous hydrolysis would occur. Thus, to confirm the metabolic pathway, we

prepared epoxycarvone (**28**) of (*R*)-(-)-carvone with *m*-CPBA as a mixture of diastereomers (1:1) (Arnone et al., 1996; Bohe and Kammonun, 2002, 2004; Smitt and Hobberg, 2002) (Scheme 6b). The epoxide (**28**) was then administrated to beetle larvae . As a result, diastereomeric diols (4*R*,8*R*)- and (4*R*,8*S*)-8,9-dihydroxy-8,9-dihydrocarvone (**26**, **27**) were produced at a ratio of 1:1 as a diastereomeric mixture (Scheme 6c). In this experiment, the epoxide appeared to be hydrolyzed easily to give diols, and no epoxide as an intermediate was detected in additional experiments.

4. Conclusion

As described above, the results of the biotransformation of organic compounds by beetle larvae (*Allomyrina dichotoma*) described in this study showed very similar reaction tendency in the case of a microorganism. It is possible that these reactions were due in part to the bacteria in the intestine of the larva; however, regio- and stereoselectivities of the reactions were sometimes unique. Thus, it is supposed that those biotransformations were accomplished by the ensemble of the larvae's own enzymes with several bacteria. Nevertheless, this is the first report on using beetle larvae as a biocatalyst and might show the possibility of using such enzymes derived from the insects as a biocatalyst.

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Author Contributions

KU and MT conceived and designed the experiments. YO, HN, KF, EH, HA, MW, and TM pereformed the experiments. KU wrote the manuscript.

Figure captions

Scheme 1. Redox of 1-phenyl-1,2-propanedione by beetle larvae.

Scheme 2. Redox of a) 1-phenyl-1,3-butanone, b) 4-phenyl-2-butanone, and 3) 1-phenyl-1-butanone by larvae of beetle.

Scheme 3. Reduction of 4-phenylbut-3-yl-2-one by beetle larvae.

Scheme 4. Reduction of acetylpyridines by beetle larvae.

Scheme 5. Redox of a) β-ionone and b) cinnamyl chloride by beetle larvae.

Scheme 6. Dihydroxylation of (*R*)-(-)-carvone by beetle larvae.

Scheme 1

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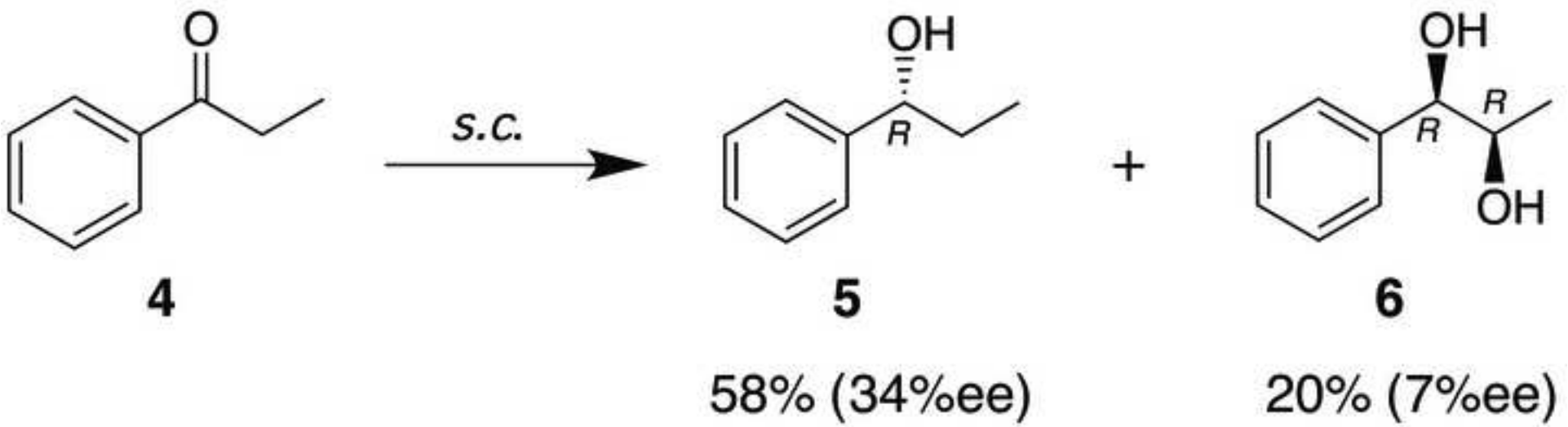

Scheme 2

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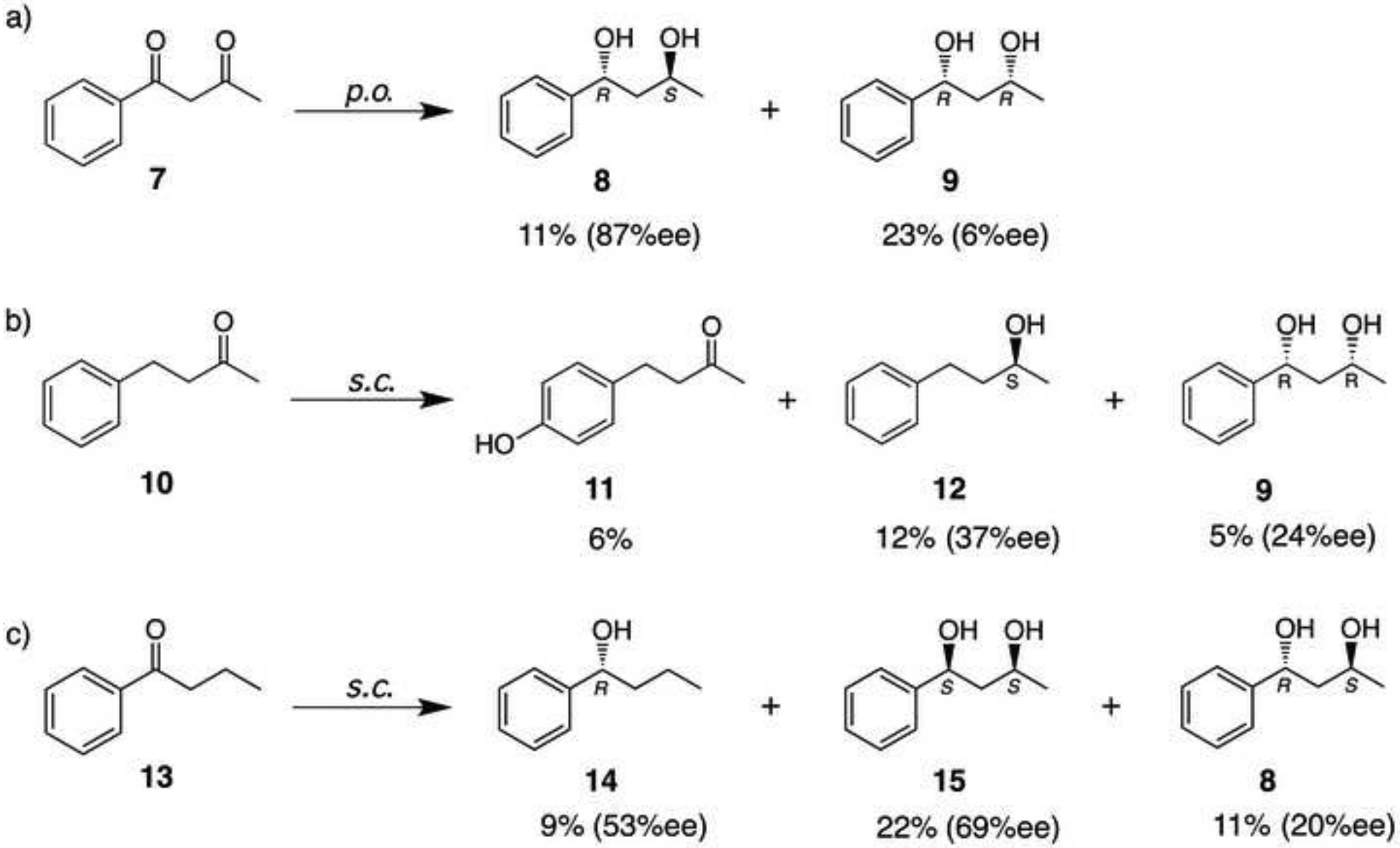

Scheme 3

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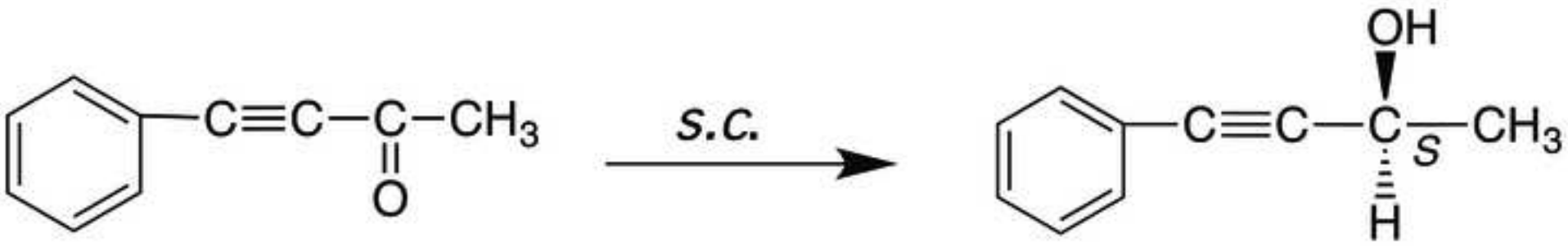

16

17

24%ee (34%)

Scheme 4

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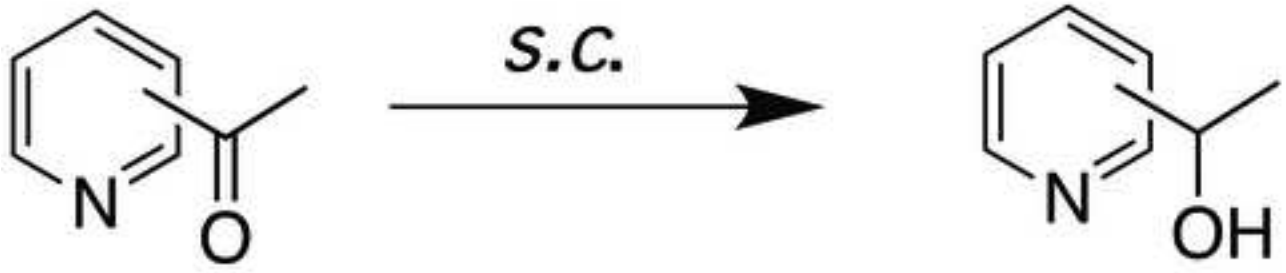

18a (α)

18b (β)

18c (γ)

19a (α) : 15% (60%ee (*S*))

19b (β) : 12% (13%ee (*R*))

19c (γ) : 5% (racemic)

Scheme 5

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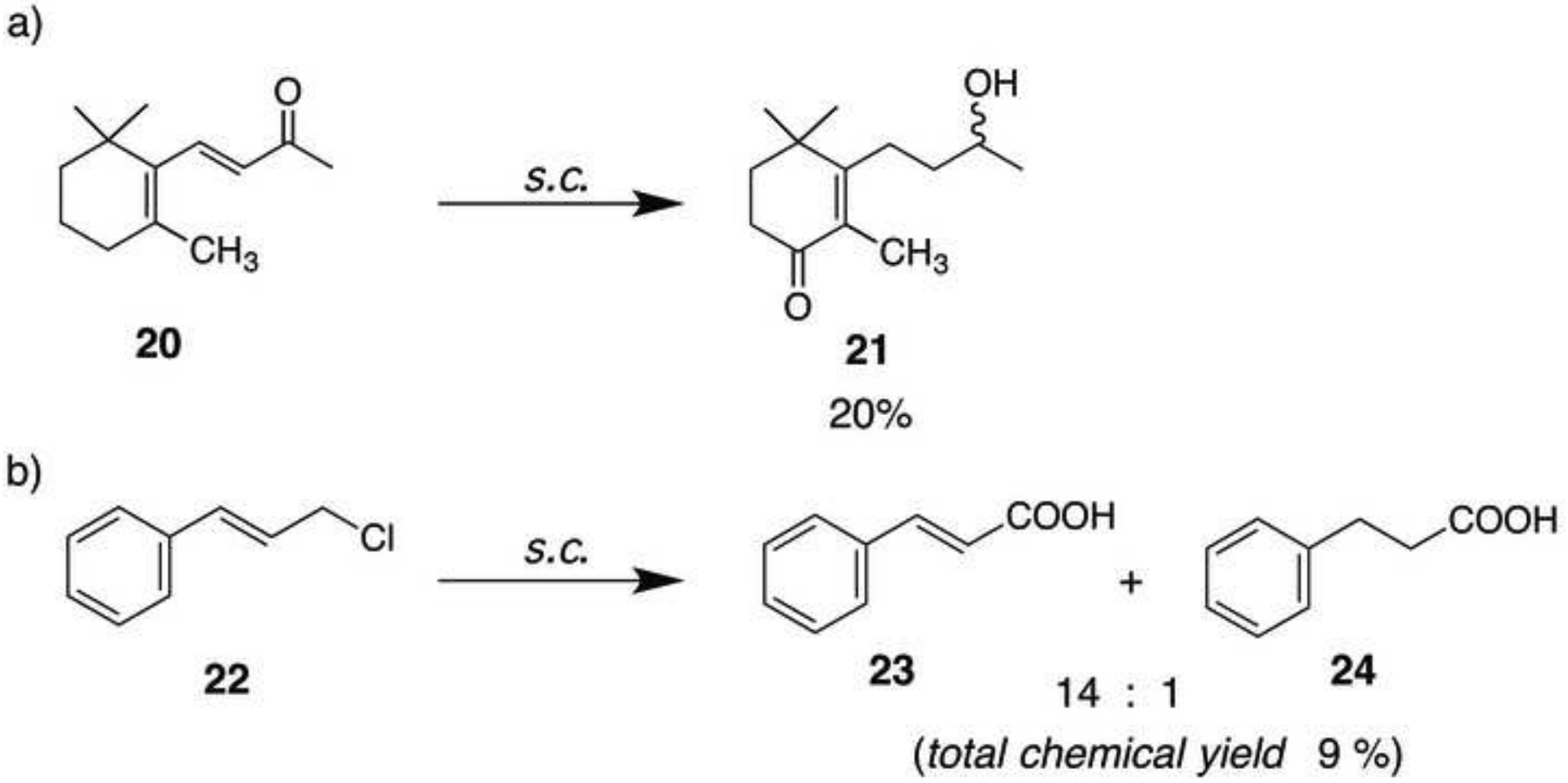

Scheme 6

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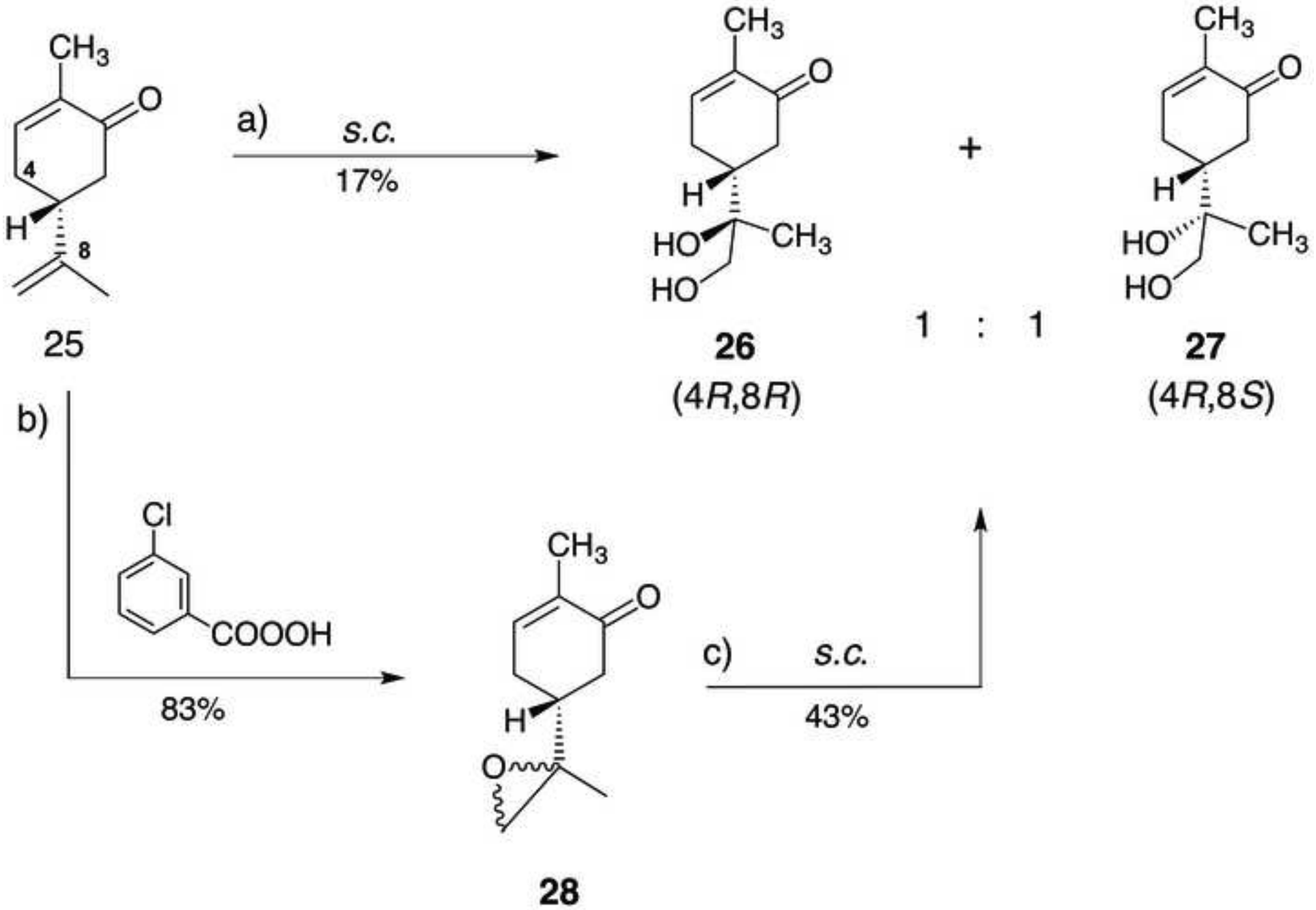

Scheme of Table 1

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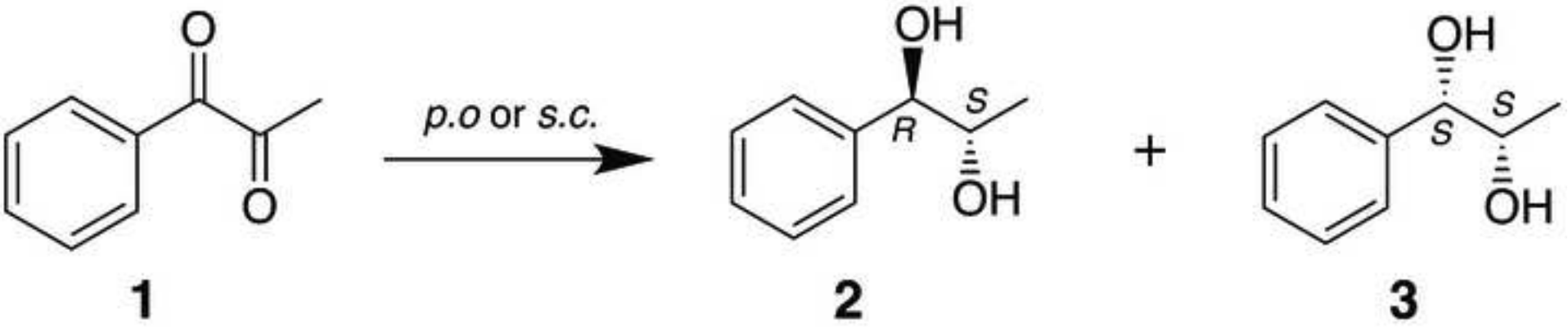

Table 1

Table 1. Reduction of 1-phenyl-1, 2-propanediones (**1**) by larvae of beetle

(Scheme of Table 1 here)

Administration	**2**		**3**	
	Yield (%)	**%ee** [a]	**Yield (%)**	**%ee** [a]
p.o.	32	92	2	92
s.c.	30	>97	2	87

[a] Determined by chiral HPLC analysis using a Daicel Chiralcel OD-H (*n*-Hexanae : 2-propanol = 85 : 15)

Graphical Abstract