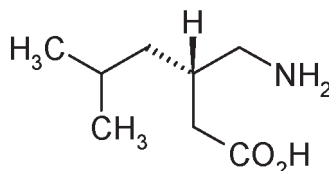


Field of the Invention

5 The present invention relates to the manufacture of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone, and derivatives thereof, for use in the manufacture of (S)-(+)-3-aminomethyl-5-methylhexanoic acid (Pregabalin). The invention further relates to an improved process for the conversion of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone into Pregabalin. Pregabalin is a γ -amino acid that exhibits binding affinity to
10 the human $\alpha_2\delta$ calcium channel subunit.

Background to the Invention

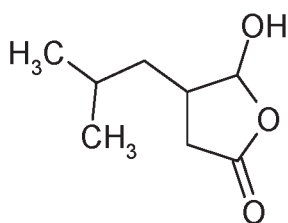
Pregabalin, or (S)-(+)-3-aminomethyl-5-methyl-hexanoic acid ((S)-II),



Pregabalin ((S)-II)

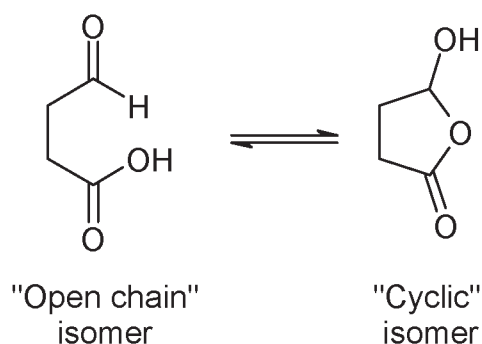
15 is the active agent in Lyrica®, which is approved for the treatment of epilepsy, neuropathic pain, fibromyalgia and generalized anxiety disorder. It exhibits anti-seizure activity, as discussed in U.S. Patent US 5,563,175, and anti-nociceptive activity, as discussed in U.S. Patent US 6,001,876. It is hypothesised that the pharmacological
20 activity of Pregabalin (II) is the result of binding to the alpha-2-delta ($\alpha_2\delta$) subunit of a calcium channel. Pregabalin (II) is also described as having utility in other conditions, such as physiological conditions associated with psychomotor stimulants, inflammation, gastrointestinal damage, alcoholism, insomnia, and various psychiatric disorders, including anxiety, depression, mania, and bipolar disorder.

25 A number of methods for manufacturing Pregabalin have been disclosed. 5-Hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone (I^A) has been identified as a useful precursor.



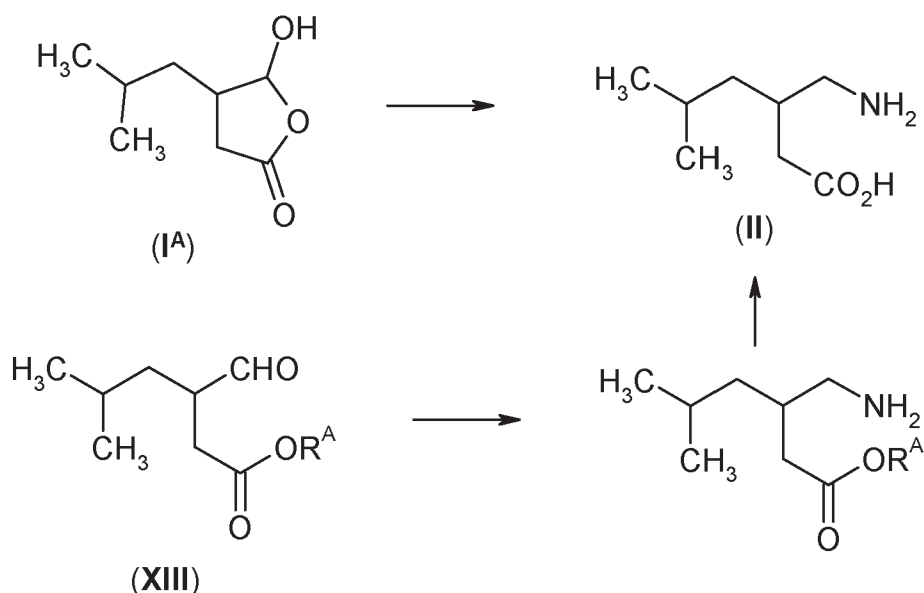
(IA)

It will be appreciated that compound (IA), in common with a number of the other compounds discussed herein, can be considered to be the cyclized isomer of a 4-oxobutanoic acid derivative. Derivatives of 4-oxobutanoic acid can exist as either the open-chain form, or as the cyclic form.

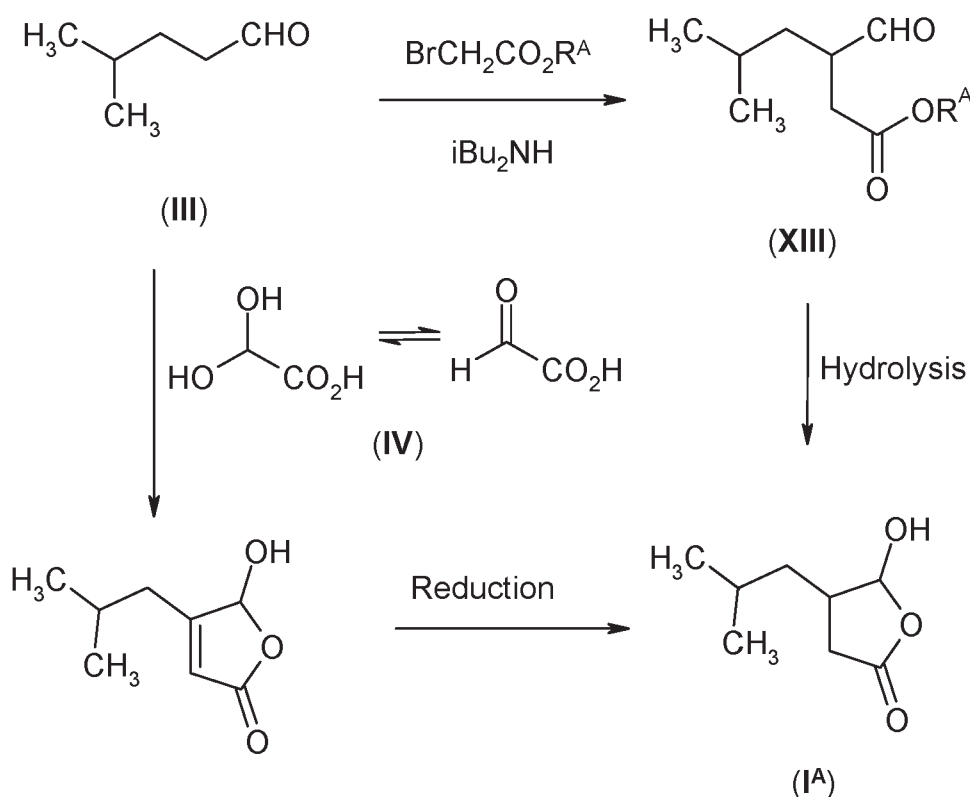


These two isomeric forms may co-exist in equilibrium, and the relative contributions of the two forms will depend on the precise chemical nature of the species.

- 10 International Patent Application PCT/US2008/004699 (published as WO2008/127646A2) proposes the conversion of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone (IA) or an ester of the ring-opened isomeric form (XIII, wherein R^A is alkyl) to (II) using a chemical or an enzyme-mediated reductive amination. It is suggested that the use of a transaminase enzyme will provide selectively the preferred
- 15 ((S)-II) enantiomer.

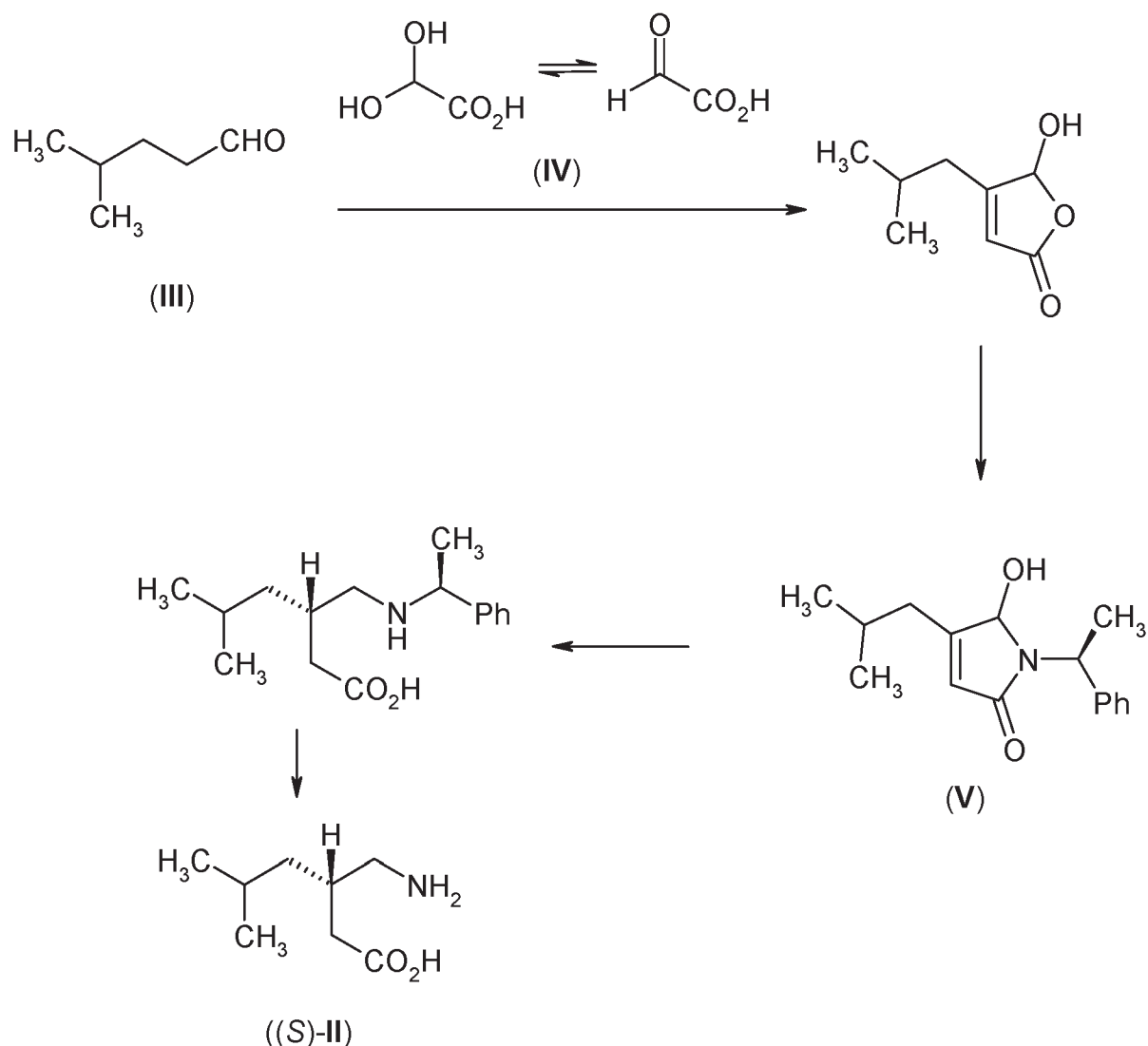


The ester (XIII) is prepared from 4-methylpentanal (III) by alkylation with an appropriate haloacetate in the presence of diisobutylamine. The precursor (IA) is made either by ester hydrolysis of ester (XIII) or by condensation of 4-methylpentanal (III) with glyoxylic acid (IV) and subsequent reduction of the double bond. The use of an enzyme-mediated reduction is also suggested as a way of introducing the desired stereochemistry.



International Patent Application PCT/IN2010/000140 (published as WO2011/086565) discloses a related process. The condensation product of 4-methylpentanal (III) and glyoxylic acid (IV) is reacted with a chiral amine such as α -methylbenzylamine to give a

pyrrolone (V). Hydrogenation gives some degree of stereoselectivity, and deprotection gives Pregabalin (II) in chiral form.

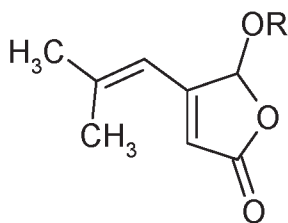


Still further improved syntheses of Pregabalin (II) are sought. It is especially desirable to provide a process which is cost effective and safe. In particular, it is important to provide a synthesis of Pregabalin (II) which can be carried out on a commercial scale, which uses readily available, inexpensive and safe starting materials and reagents, and which avoids the need for difficult separations.

10 Summary of the Invention

The present invention provides improved methods for the manufacture of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone (**I^A**), and intermediates that are useful in these improved methods.

15 In a first aspect **A1**, the invention provides a compound according to formula (**VI**)



(VI)

In a first embodiment **A1E1**, the invention provides a compound according to formula (VI) wherein R is selected from:

- 5 hydrogen,
(C₁-C₆)alkyl,
(C₁-C₆)haloalkyl,
(C₁-C₃)alkoxy(C₂-C₆)alkyl,
(C₂-C₆)alkenyl,
10 (C₃-C₁₀)cycloalkyl, which may optionally be substituted with 1, 2 or 3 groups
independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,
(C₃-C₁₀)cycloalkyl(C₁-C₆)alkyl, wherein the cycloalkyl group may optionally be
substituted with 1, 2 or 3 groups independently selected from halo, (C₁-
C₃)alkyl, and (C₁-C₃)alkyloxy,
15 aryl, which may optionally be substituted with 1, 2 or 3 groups independently
selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,
aryl(C₁-C₆)alkyl, wherein the aryl group may optionally be substituted with 1, 2 or
3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-
C₃)alkyloxy,
20 R¹-C(O)-, and
R²-SO₂-;

R^1 is selected from:

- hydrogen,
(C₁-C₆)alkyl,
25 (C₁-C₆)haloalkyl,
(C₁-C₃)alkoxy(C₂-C₆)alkyl,
(C₂-C₆)alkenyl,
(C₃-C₁₀)cycloalkyl, which may optionally be substituted with 1, 2 or 3 groups
independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,

(C₃-C₁₀)cycloalkyl(C₁-C₆)alkyl, wherein the cycloalkyl group may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,

aryl, which may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy, and

aryl(C₁-C₆)alkyl, wherein the aryl group may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,

and R² is selected from:

(C₁-C₆)alkyl,

(C₁-C₆)haloalkyl,

(C₁-C₃)alkoxy(C₂-C₆)alkyl,

(C₂-C₆)alkenyl,

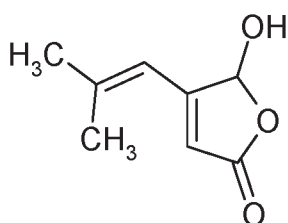
(C₃-C₁₀)cycloalkyl, which may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,

(C₃-C₁₀)cycloalkyl(C₁-C₆)alkyl, wherein the cycloalkyl group may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy,

aryl, which may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy, and

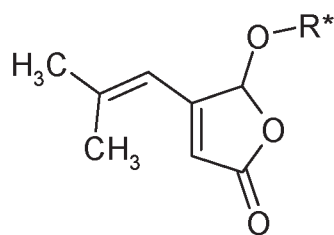
aryl(C₁-C₆)alkyl, wherein the aryl group may optionally be substituted with 1, 2 or 3 groups independently selected from halo, (C₁-C₃)alkyl, and (C₁-C₃)alkyloxy.

In a further embodiment **A1E2**, the invention provides a compound according to embodiment **A1E1** wherein R is hydrogen such that the compound of formula (VI) is 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone according to formula (VI^A).



(VI^A)

In a further embodiment **A1E3**, the invention provides a compound according to embodiment **A1E1** of formula (**VI^B**)

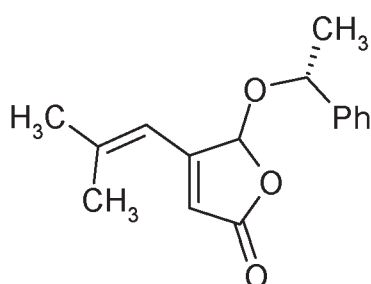


(**VI^B**)

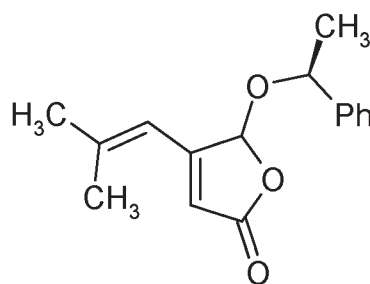
wherein R* is a chiral (C₅-C₁₅) hydrocarbon group.

5

In a further embodiment **A1E4**, the invention provides a compound according to embodiment **A1E3** wherein R* is selected from (*R*)- or (*S*)- α -methylbenzyl, (*R*)- or (*S*)-1-(1-naphthyl)ethyl, (*R*)- or (*S*)-1-(2-naphthyl)ethyl, menthyl and bornyl, such that the compound of formula (**VI**) is the compound of formula (**VI^C**) - (**VI^K**).

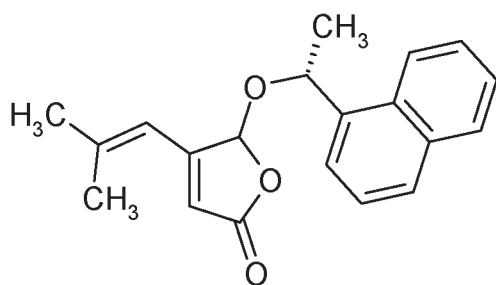


(**VI^C**)

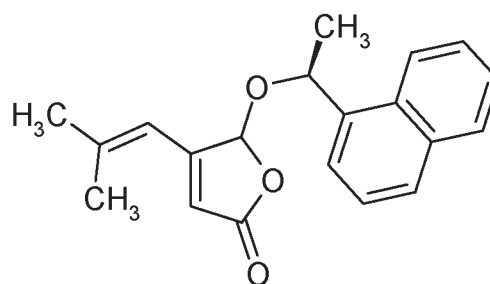


(**VI^D**)

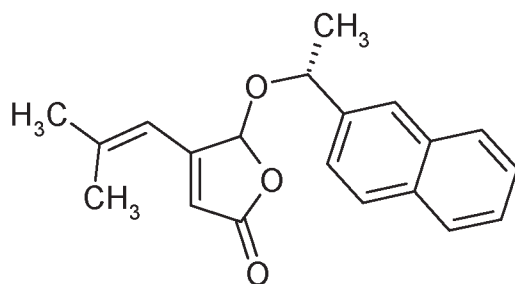
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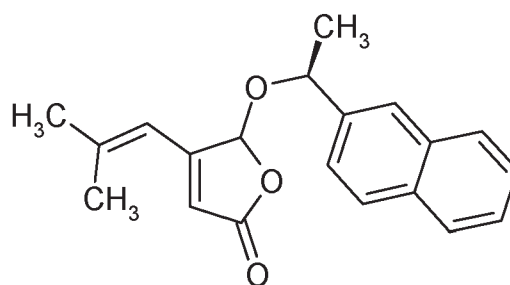
(**VI^E**)



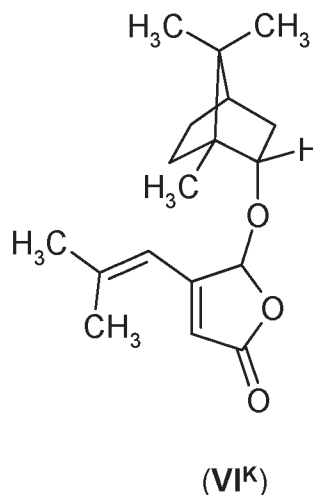
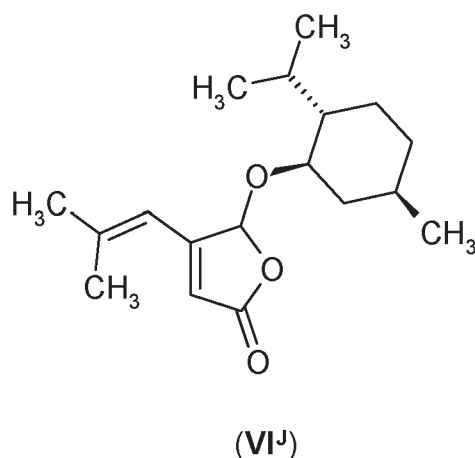
(**VI^F**)



(**VI^G**)

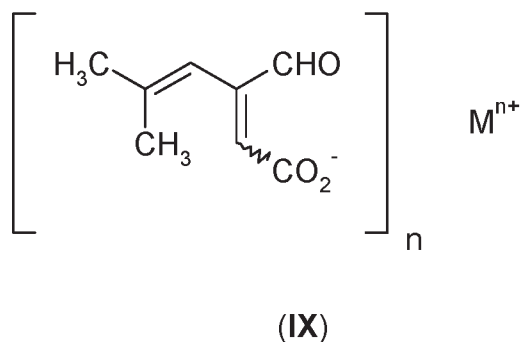


(**VI^H**)



In a further embodiment **A1E5**, the invention provides a compound according to
 5 embodiment **A1E1** wherein R is R¹-C(O)- or R²-SO₂- and R¹ and R² are chiral (C₅-C₁₅) hydrocarbon groups.

In another aspect **A2**, the invention provides a compound of formula (IX)



10

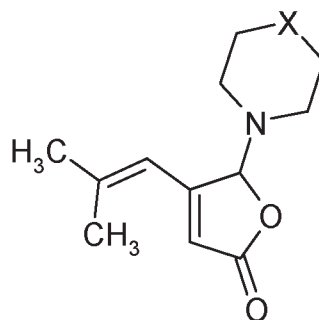
The compound of formula (IX) may exist as either the (*E*)- or (*Z*)-geometric isomer, or as a mixture of the two geometric isomers.

In a first embodiment **A2E1**, the invention provides a compound of formula (IX) wherein:
 15 n is 1 and M⁺ is selected from Li⁺, Na⁺, K⁺, Rb⁺, NH₄⁺, ((C₁-C₃)alkyl)NH₃⁺, ((C₁-C₃)alkyl)₂NH₂⁺, ((C₁-C₃)alkyl)₃NH⁺ and ((C₁-C₃)alkyl)₄N⁺; or
 n is 2 and M²⁺ is selected from Mg²⁺, Ca²⁺ and Zn²⁺.

In a further embodiment **A2E2**, the invention provides a compound according to
 20 embodiment **A2E1** wherein n is 1 and M⁺ is selected from NH₄⁺ and ((C₁-C₃)alkyl)NH₃⁺.

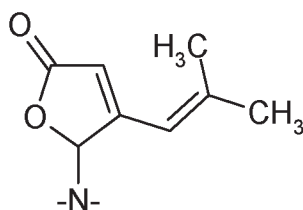
In a further embodiment **A2E3**, the invention provides a compound according to embodiment **A2E1** wherein n is 1 and M^+ is selected from Li^+ , Na^+ and K^+ .

In another aspect **A3**, the invention provides a compound of formula (VII).



(VII)

In a first embodiment **A3E1**, the invention provides a compound of formula (VII) wherein $-X-$ represents a single bond, $-CH_2-$, $-O-$, $-NH-$, $-N((C_1-C_3)alkyl)-$, $-N(benzyl)-$, or



In a further embodiment **A3E2**, the invention provides a compound according to embodiment **A3E1** selected from:

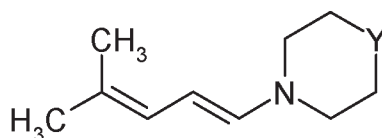
4-(2-methylpropenyl)-5-pyrrolidin-1-yl-5H-furan-2-one;

4-(2-methylpropenyl)-5-piperidin-1-yl-5H-furan-2-one;

15 4-(2-methylpropenyl)-5-morpholin-4-yl-5H-furan-2-one; and

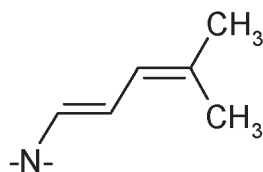
1,4-bis-(4-(2-methylpropenyl)-5H-furan-2-on-5-yl)piperazine.

In another aspect **A4**, the invention provides a compound of formula (VIII).



(VIII)

In a first embodiment **A4E1**, the invention provides a compound of formula (VIII) wherein -Y- represents a single bond, -CH₂-, -O-, -NH-, -N((C₁-C₃)alkyl)-, -N(benzyl)-, or



5

In a further embodiment **A4E2**, the invention provides a compound according to embodiment **A4E1** selected from:

4-(4-methyl-1,3-pentadien-1-yl)morpholine;

1-(4-methyl-1,3-pentadien-1-yl)-piperazine,

10 1-(4-methyl-1,3-pentadien-1-yl)-4-methylpiperazine,

4-ethyl-1-(4-methyl-1,3-pentadien-1-yl)-piperazine,

4-benzyl-1-(4-methyl-1,3-pentadien-1-yl)-piperazine, and

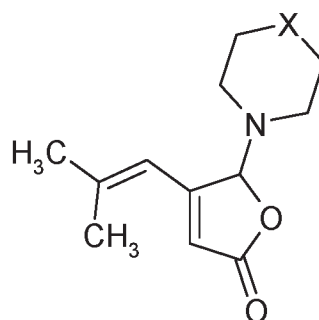
1,4-*bis*-(4-methyl-1,3-pentadien-1-yl)piperazine.

15 In another aspect **A5**, the invention provides a process for the manufacture of the compound of formula (VI^A) comprising the step of treating a compound of formula (VII) with water in the presence of an acid catalyst.

In a first embodiment **A5E1**, the invention provides a process for the manufacture of the compound of formula (VI^A), comprising the steps of:

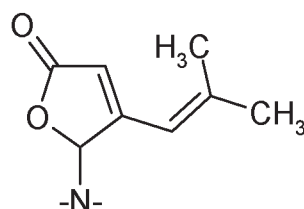
20

(a) preparing a compound of formula (VII)



(VII)

wherein -X- represents a single bond, -CH₂-, -O-, -NH-, -N((C₁-C₃)alkyl)-, -N(benzyl)-, or



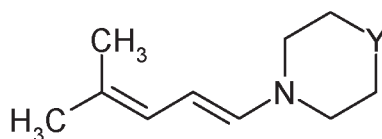
and

(b) treating the compound of formula (VII) with water in the presence of an acid catalyst.

In a further embodiment **A5E2**, the invention provides a process according to embodiment **A5E1** wherein the compound of formula (VII) from step (a) is isolated prior to hydrolysis step (b).

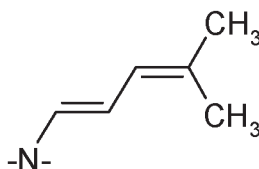
In a further embodiment **A5E3**, the invention provides a process according to embodiment **A5E1** wherein hydrolysis step (b) is carried out directly following step (a) such that the compound of formula (VII) or (VII^B) is not isolated prior to hydrolysis step (b).

In a further embodiment **A5E4**, the invention provides a process according to embodiments **A5E1**, **A5E2** or **A5E3** wherein the compound of formula (VII) is prepared by treating a compound of formula (VIII)



(VIII)

wherein -Y- represents a single bond, -CH₂-, -O-, -NH-, -N((C₁-C₃)alkyl)-, -N(benzyl)-, or



with glyoxylic acid or its hydrate.

In another aspect **A6**, the invention provides a process for the manufacture of a compound of formula (VI) wherein R is other than hydrogen, $R^1-C(O)-$, and R^2-SO_2- , comprising the step of treating a compound of formula (VI^A) with an alcohol R-OH in the presence of an acid catalyst.

5

In an embodiment **A6E1**, the invention provides a process for the manufacture of a compound of formula (VI) wherein R is other than hydrogen, $R^1-C(O)-$, and R^2-SO_2- , comprising the steps of:

- (a) preparing a compound of formula (VI^A) using a process according to any of
10 embodiments **A5E1**, **A5E2**, **A5E3** and **A5E4** as defined above; and
(b) treating the compound of formula (VI^A) with an alcohol R-OH in the presence of
 an acid catalyst.

In another aspect **A7**, the invention provides a process for the manufacture of a
15 compound of formula (VI) wherein R is other than hydrogen, $R^1-C(O)-$, and R^2-SO_2- ,
 comprising the step of treating a compound of formula (VII) with an alcohol R-OH in the
 presence of stoichiometric acid.

In an embodiment **A7E1**, the invention provides a process for the manufacture of a
20 compound of formula (VI) wherein R is other than hydrogen, $R^1-C(O)-$, and R^2-SO_2- ,
 comprising the steps of:

- (a) preparing a compound of formula (VII); and
(b) treating the compound of formula (VII) with an alcohol R-OH in the presence of
 stoichiometric acid.

25

In another aspect **A8**, the invention provides a process for the manufacture of a
 compound of formula (VI) wherein R is $R^1-C(O)-$, comprising the step of treating a
 compound of formula (VI^A) with an acid chloride $R^1-C(O)-Cl$ or acid anhydride $(R^1-C(O))_2O$.

30

In an embodiment **A8E1**, the invention provides a process for the manufacture of a
 compound of formula (VI) wherein R is $R^1-C(O)-$, comprising the steps of:

- (a) preparing a compound of formula (VI^A) using a process according to any of
 embodiments **A5E1**, **A5E2**, **A5E3** and **A5E4** as defined above; and

(b) treating the compound of formula (VI^A) with an acid chloride R¹-C(O)-Cl or acid anhydride (R¹-C(O))₂O.

In another aspect **A9**, the invention provides a process for the manufacture of a compound of formula (VI) wherein R is R²-SO₂-, comprising the step of treating a compound of formula (VI^A) with a sulfonyl chloride R²-SO₂-Cl.

In an embodiment **A9E1**, the invention provides a process for the manufacture of a compound of formula (VI) wherein R is R²-SO₂-, comprising the steps of:

- (a) preparing a compound of formula (VI^A) using a process according to any of embodiments **A5E1**, **A5E2**, **A5E3** and **A5E4** as defined above; and
- (b) treating the compound of formula (VI^A) with a sulfonyl chloride R²-SO₂-Cl.

In another aspect **A10**, the invention provides a process for the manufacture of an enamine derivative of 4-methyl-2-pentenal.

In a first embodiment **A10E1**, the invention provides a process for the manufacture of an enamine derivative of 4-methyl-2-pentenal comprising reacting acetaldehyde with isobutyraldehyde in the presence of a suitable amine.

In a further embodiment **A10E2**, the invention provides a process according to embodiment **A10E1** wherein the suitable amine is a secondary amine.

In a further embodiment **A10E3**, the invention provides a process according to embodiment **A10E2** wherein the secondary amine is selected from: ((C₁-C₄)alkyl)₂NH, pyrrolidine, piperidine, morpholine, piperazine, N-methylpiperazine, N-ethylpiperazine and N-benzylpiperazine.

In a further embodiment **A10E4**, the invention provides a process according to embodiment **A10E3** wherein the secondary amine is selected from pyrrolidine, piperidine, morpholine, and piperazine.

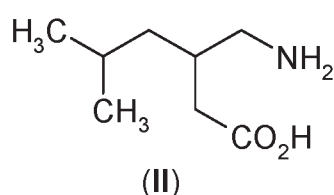
In a further embodiment **A10E5**, the invention provides a process according to any of embodiments **A10E1**, **A10E2**, **A10E3** and **A10E4** wherein the reaction is performed in the presence of an acid catalyst.

In a further embodiment **A10E6**, the invention provides a process according to any of embodiments **A10E1**, **A10E2**, **A10E3**, **A10E4** and **A10E5** wherein the isobutyraldehyde is combined with the suitable amine before addition of the acetaldehyde.

5

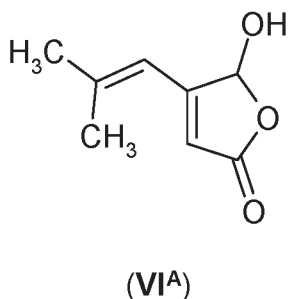
In a further embodiment **A10E6**, the invention provides a process according to any of embodiments **A10E1**, **A10E2**, **A10E3**, **A10E4** and **A10E5** wherein the isobutyraldehyde and acetaldehyde are added to the reaction vessel simultaneously.

- 10 In another aspect **A11**, the invention provides a process for the manufacture of 3-aminomethyl-5-methylhexanoic acid (**II**).

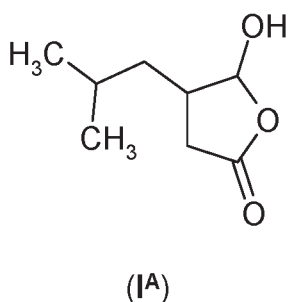


- In a first embodiment **A11E1**, the invention provides a process for the manufacture of 3-aminomethyl-5-methylhexanoic acid (**II**) or a pharmaceutically acceptable salt thereof,
- 15 comprising the steps:

(a) preparing 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**)



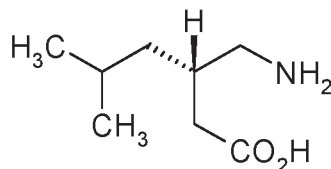
- (b) converting said 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**) into 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone (**I^A**)
- 20



and

(c) converting said 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone (**I^A**) into 3-aminomethyl-5-methylhexanoic acid (**II**).

In a further embodiment **A11E2**, the invention provides a process according to embodiment **A11E1** wherein the 3-aminomethyl-5-methylhexanoic acid (**II**) is (*S*)-3-aminomethyl-5-methylhexanoic acid ((*S*)-**II**)



((*S*)-**II**)

wherein said (*S*)-3-aminomethyl-5-methylhexanoic acid has an enantiomeric excess of at least 80%.

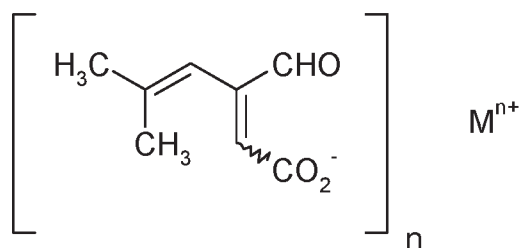
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In a further embodiment **A11E3**, the invention provides a process according to embodiment **A11E1** or **A11E2** wherein step (a) comprises a process according to embodiments **A5E1**, **A5E2**, **A5E3** or **A5E4** as described above.

15 In a further embodiment **A11E4**, the invention provides a process according to embodiment **A11E1**, **A11E2** or **A11E3** wherein step (b) comprises the steps:

(b1) treating the 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**) with a metal oxide, hydroxide, carbonate or bicarbonate, ammonia, a mono- di- or tri-(*C*₁-*C*₃)alkylamine, or a tetra-(*C*₁-*C*₃)alkylammonium hydroxide to form a salt of formula (**IX**)

20



(**IX**)

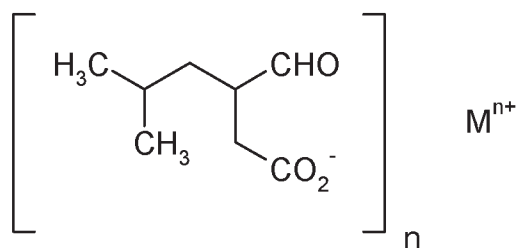
wherein:

n is 1 and *M*⁺ is selected from Li⁺, Na⁺, K⁺, Rb⁺, NH₄⁺, ((*C*₁-*C*₃)alkyl)NH₃⁺, ((*C*₁-*C*₃)alkyl)₂NH₂⁺, ((*C*₁-*C*₃)alkyl)₃NH⁺ and ((*C*₁-*C*₃)alkyl)₄N⁺; or

25

n is 2 and *M*²⁺ is selected from Mg²⁺, Ca²⁺ and Zn²⁺;

(b2) hydrogenating the salt of formula (**IX**) to obtain a salt of formula (**X**)



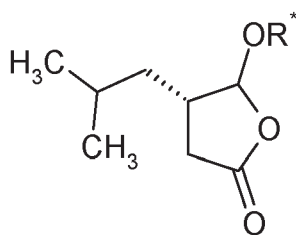
(X) ; and

(b3) treating the salt of formula (X) with an acid.

In a further embodiment **A11E5**, the invention provides a process according to embodiment **A11E1**, **A11E2** or **A11E3** wherein step (b) comprises the steps:

(b1) converting the 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**) to a compound of formula (**VI**) as defined in embodiment **A1E3**, wherein R is a chiral (C₅-C₁₅)hydrocarbon group;

(b2) hydrogenating the compound of formula (**VI**) to obtain a compound of formula (**XI**)



(XI)

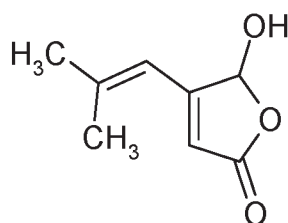
wherein R* is a chiral (C₅-C₁₅)hydrocarbon group; and

(b3) treating the compound of formula (**XI**) with an acid to give ((S)-I^A).

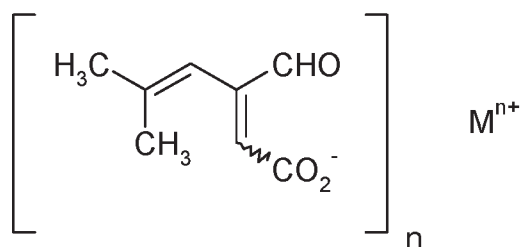
15 In another aspect **A12**, the invention provides a further process for the manufacture of 3-aminomethyl-5-methylhexanoic acid (**II**).

In a first embodiment **A12E1**, the invention provides a process for the manufacture of 3-aminomethyl-5-methylhexanoic acid (**II**) or a pharmaceutically acceptable salt thereof, comprising the steps:

(a) preparing 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**)

(VI^A)

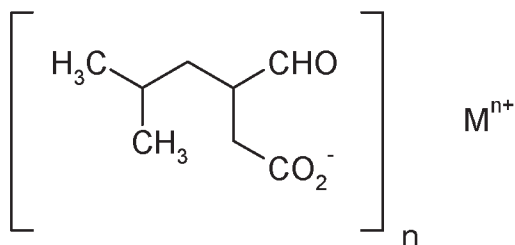
(b) treating the 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (VI^A) with ammonia or a mono-(C₁-C₃)alkylamine to form a salt of formula (IX^A)

(IX^A)

5

wherein *n* is 1 and M⁺ is selected from NH₄⁺ and ((C₁-C₃)alkyl)NH₃⁺

(c) hydrogenating the salt of formula (IX^A) to obtain a salt of formula (X^A)

(X^A)

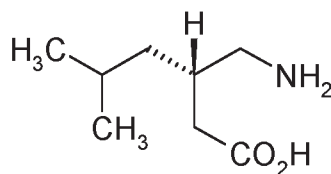
; and

10

(d) treating the salt of formula (X^A) with a transaminase or an amine oxidase/imine reductase enzyme to provide 3-aminomethyl-5-methylhexanoic acid (II).

15

In a further embodiment **A12E2**, the invention provides a process according to embodiment **A12E1** wherein the 3-aminomethyl-5-methylhexanoic acid (II) is (S)-3-aminomethyl-5-methylhexanoic acid ((S)-II)



((S)-II)

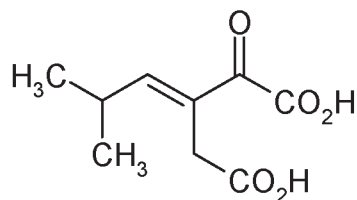
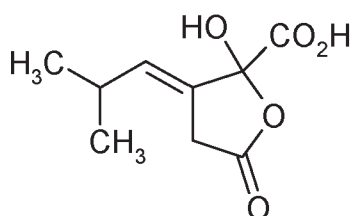
wherein said (S)-3-aminomethyl-5-methylhexanoic acid has an enantiomeric excess of at least 80%.

- 5 In a further embodiment **A12E3**, the invention provides a process according to embodiment **A12E1** or **A12E2** wherein step (a) comprises a process according to embodiments **A5E1**, **A5E2**, **A5E3** or **A5E4** as described above.

In another aspect **A13**, the invention provides a further process for the manufacture of
10 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone (**I^A**).

In a first embodiment **A13E1**, the invention provides a further process for the manufacture of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone (**I^A**) which comprises the steps of:

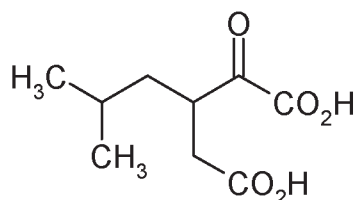
- 15 (a) obtaining 3-isobutylidene-2-oxopentanedioic acid (**XII^A**) or its cyclised isomer (**XII^B**)

(XII^A)(XII^B)

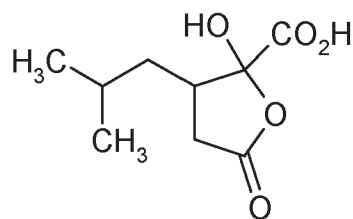
, and

- (b) sequentially or simultaneously reducing the carbon-carbon double bond and
20 decarboxylating the α -keto acid functional group.

In a further embodiment **A13E2**, the invention provides a process according to embodiment **A13E1** wherein the carbon-carbon double bond is reduced to provide 3-isobutyl-2-oxopentanedioic acid (**XV**) or its cyclised isomer (**XV^A**)

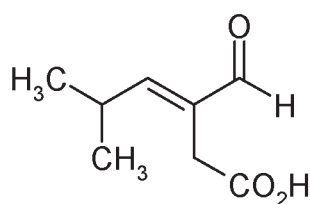


(XV)

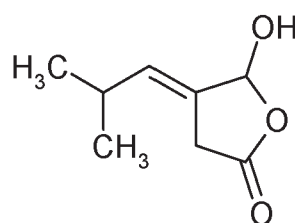
(XV^A)

before the decarboxylation of the α -keto acid functional group.

In a further embodiment **A13E3**, the invention provides a process according to
 5 embodiment **A13E1** wherein the α -keto acid functional group is decarboxylated to
 provide 3-formyl-5-methyl-3-pentenoic acid (**XVI** or its cyclised isomer (**XVI^A**)



(XVI)

(XVI^A)

before the reduction of the carbon-carbon double bond.

10 In a further embodiment **A13E4**, the invention provides a process according to
 embodiment **A13E1** wherein the α -keto acid functional group is decarboxylated and the
 carbon-carbon double bond is reduced simultaneously.

In a further embodiment **A13E5**, the invention provides a process according to
 15 embodiments **A13E1**, **A13E2**, **A13E3** or **A13E4** wherein the decarboxylation is carried
 out in the presence of a decarboxylase enzyme.

In a further embodiment **A13E6**, the invention provides a process according to
 embodiments **A13E1**, **A13E2**, **A13E3**, **A13E4** or **A13E5** wherein the reduction of the
 20 carbon-carbon double bond is carried out in the presence of an enoate reductase
 enzyme.

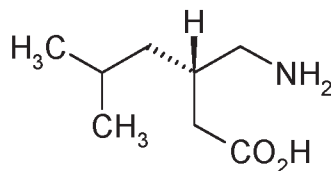
In another aspect **A14**, the invention provides a compound selected from:

3-isobutylidene-2-oxopentanedioic acid;

25 3-isobutyl-2-oxopentanedioic acid; and

3-formyl-5-methyl-3-pentenoic acid,
or a salt, (C₁-C₆)alkyl ester or cyclised isomer thereof.

In another aspect **A15**, the invention provides a process for the manufacture of (S)-3-aminomethyl-5-methylhexanoic acid ((S)-II)



((S)-II)

or a pharmaceutically acceptable salt thereof, comprising the steps:

- (a) manufacturing 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone (**I^A**) using a process according to any one of embodiments **A13E1**, **A13E2**, **A13E3**, **A13E4**, **A13E5** or **A13E6** as defined above, and
- (b) converting said 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone into (S)-3-aminomethyl-5-methylhexanoic acid.

In another aspect **A16**, the invention provides a process for converting (R)-3-aminomethyl-5-methylhexanoic acid into (S)-3-aminomethyl-5-methylhexanoic acid comprising treating the (R)-3-aminomethyl-5-methylhexanoic acid with a transaminase enzyme or an amine oxidase/imine reductase enzyme.

In another aspect **A17**, the invention provides a process for increasing the proportion of (S)-3-aminomethyl-5-methylhexanoic acid in a mixture of (R)- and (S)-3-aminomethyl-5-methylhexanoic acid comprising treating the mixture with a transaminase enzyme or an amine oxidase/imine reductase enzyme.

In another aspect **A18**, the invention provides a transaminase enzyme that is useful for the conversion of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone (**I^A**) into Pregabalin.

In a first embodiment **A18E1**, the invention provides a transaminase enzyme having an amino acid sequence that has at least 95% homology to the amino acid sequence

MNKPQSWEARAETYSLYGFTDMPSLH X^{27} RGTVVVTHGEGPY X^{41} VDV X^{45} GRRYLDAN
 SGLYNMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGR
 VFYTNSGSEANDTMVKMLWFLHAAEGKPQKRKILTR X^{147} NAYHGVTAVSASMTG X^{163} P
 X^{165} NSVFGLPLPGFVHL X^{180} CPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAG
 5 FFAEPVMGAGGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPD
 AISSKNLTAGFFPVGAVILGPEL X^{304} KRLETAIEAIEEFPHGFT X^{324} GHPVGCAIALKAID
 VVMNEGLAENVRRRLAPRFEERLKHIAERPNIIGEYRGIGFMWALEAVKDKASKTPFDGN
 LSVS X^{401} RIANTC X^{408} DLGLIC X^{415} X^{416} X^{417} GQSVIL X^{424} PPFILTEAQMDDEMFDKLEKALD
 KVFAEVA (SEQ ID NO. 1)

10

wherein

X^{27} is selected from glutamine (Q) and glutamic acid (E);

X^{41} is selected from isoleucine (I) and valine (V);

X^{45} is selected from asparagine (N) and histidine (H);

15 X^{147} is selected from asparagine (N) and glutamine (Q);

X^{163} is selected from leucine (L) and methionine (M);

X^{165} is selected from tyrosine (Y) and histidine (H);

X^{180} is selected from threonine (T); glycine (G) and serine (S);

X^{304} is selected from alanine (A) and serine (S);

20 X^{324} is selected from glycine (G) and serine (S);

X^{401} is selected from lysine (K) and glutamic acid (E);

X^{408} is selected from threonine (T) and glutamine (Q);

X^{415} is selected from serine (S) and alanine (A);

X^{416} is selected from proline (P) and alanine (A);

25 X^{417} is selected from leucine (L) and methionine (M); and

X^{424} is selected from cysteine (C) and serine (S).

In a further embodiment **A18E2**, the invention provides a transaminase enzyme according to embodiment **A18E1** having the amino acid sequence of SEQ ID NO. 1.

30

In a further embodiment **A18E3**, the invention provides a transaminase enzyme according to embodiment **A18E1** or **A18E2** wherein:

X^{27} is glutamic acid (E);

X^{147} is glutamine (Q);

35 X^{165} is histidine (H);

X³⁰⁴ is serine (S);

X³²⁴ is glycine (G);

X⁴⁰¹ is lysine (K);

X⁴⁰⁸ is glutamine (Q);

5 X⁴¹⁶ is alanine (A);

X⁴¹⁷ is methionine (M); and

X⁴²⁴ is serine (S).

10 In a further embodiment **A18E4**, the invention provides a transaminase enzyme according to embodiment **A18E2** having an amino acid sequence selected from:

MNKPQSWEARAETYSLYGFTDMPSLHQRGTVVVTHGEGPYIVDVNGRRYLDANGL
YNMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVFY
TNSGSEANDTMVKMLWFLHAAEGKPQKRKILTRNNAYHGVTAVSASMTGLPYNSVFG
15 LPLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAISSKNLTA
GFFPVGAVILGPELAKRLETAIEAIEEFPHGFTASGHPVGCAIALKAIDVVMNEGLAENV
RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSERIANTCT
DLGLICSPMGQSVILCPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 2);

20 MNKPQSWEARAETYSLYGFTDMPSLHERGTVVVTHGEGPYIVDVNGRRYLDANGLY
NMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVFYT
NSGSEANDTMVKMLWFLHAAEGKPQKRKILTRNNAYHGVTAVSASMTGLPYNSVFG
PLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGAG
25 GVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAISSKNLTAG
FFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
RLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQD
LGLICSALGQSVILCPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 3);

30 MNKPQSWEARAETYSLYGFTDMPSLHERGTVVVTHGEGPYVVDVNGRRYLDANGL
YNMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVFY
TNSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGLPHNSVFG
LPLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAISSKNLTA
35 GFFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV

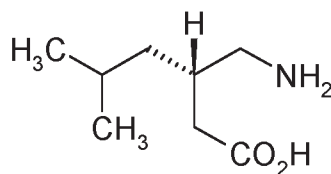
RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
DLGLICSALGQSVILSPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 4);

MNKPQSWEARAETYSLYGFTDMPSLHERGTWVTHGEGPYIVDVNGRRYLDANSGLY
5 NMVAGFDHKGLIDA AKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFD SGRVFYT
NSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGMPHNSVFG
LPLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAIIS SKNLTA
GFFPVGAVILGPALSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
10 RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
DLGLICSAMGQSVILSPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 5);

MNKPQSWEARAETYSLYGFTDMPSLHERGTWVTHGEGPYVVDVNGRRYLDANSGL
YNMVAGFDHKGLIDA AKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFD SGRVFY
15 TNSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGLPHNSVFG
LPLPGFVHLGCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAIIS SKNLTA
GFFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
20 DLGLICAAMGQSVILSPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 6); and

MNKPQSWEARAETYSLYGFTDMPSLHERGTWVTHGEGPYIVDVHGRRYLDANSGLY
NMVAGFDHKGLIDA AKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFD SGRVFYT
NSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGLPHNSVFG
25 PLPGFVHLSCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGAG
GVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAIIS SKNLTA
FFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
RLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
LGLICSAMGQSVILSPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 7).

30 In another aspect **A19**, the invention provides a process for the manufacture of (S)-3-aminomethyl-5-methylhexanoic acid ((S)-II)



((S)-II)

or a pharmaceutically acceptable salt thereof, comprising the step of treating 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5*H*-2-furanone (**I^A**) and an amine with a transaminase enzyme according to any one of embodiments **A18E1**, **A18E2**, **A18E3** and **A18E4**.

5

Detailed Description of Preferred Embodiments

The term “alkyl” means a straight-chain or branched-chain saturated aliphatic hydrocarbon radical containing the specified number of carbon atoms. Examples of alkyl radicals include methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, neopentyl and *n*-hexyl.

10

The term “alkoxy” means a group made up of an alkyl group as defined above connected to an oxygen atom. Examples of alkoxy groups include methoxy, ethoxy and isopropoxy.

15

The term “alkoxy-alkyl” means a straight-chain or branched-chain saturated aliphatic hydrocarbon radical in which an alkoxy group is substituted for an alkyl hydrogen atom. An example of an alkoxy-alkyl group is 2-methoxyethyl.

20

The term “haloalkyl” means an alkyl group as defined above wherein one or more hydrogen atoms are replaced by fluorine, chlorine, bromine or iodine. When more than one hydrogen atom is replaced, the replacing halogen atoms may be the same or different. Examples of haloalkyl groups include fluoromethyl, difluoromethyl, trifluoromethyl, chlorodifluoromethyl, 2,2,2-trifluoroethyl and 3-bromopropyl.

25

The term “aryl” means a phenyl or naphthyl group.

The term “aryl-alkyl” means a straight-chain or branched-chain saturated aliphatic hydrocarbon radical in which an aryl group is substituted for an alkyl hydrogen atom.

30

An example of an aryl-alkyl group is benzyl.

The term "cycloalkyl" means a saturated monocyclic or polycyclic carbocyclic ring containing the specified number of carbon atoms. Examples of monocyclic cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl. Examples of polycyclic cycloalkyl groups include bicyclo[2,2,1]heptyl and bicyclo[3,2,1]octyl.

5

The term "optionally substituted" with reference to an alkyl or aryl group means that a hydrogen atom of the alkyl or aryl group may be replaced by one of the groups listed. The substitution may be made at any position within the alkyl or aryl group. When the optional substitution is with "one or more groups" then any number of hydrogen atoms of the alkyl or aryl group, up to a maximum equal to the number of hydrogens present in the alkyl or aryl group, may be replaced, and each replacement is independent of the others.

10

The term "enantiomeric excess", sometimes abbreviated as "e.e.", is a measure, for a given sample, of the excess of one enantiomer in excess of its antipode and is expressed as a percentage. Enantiomeric excess is defined as:

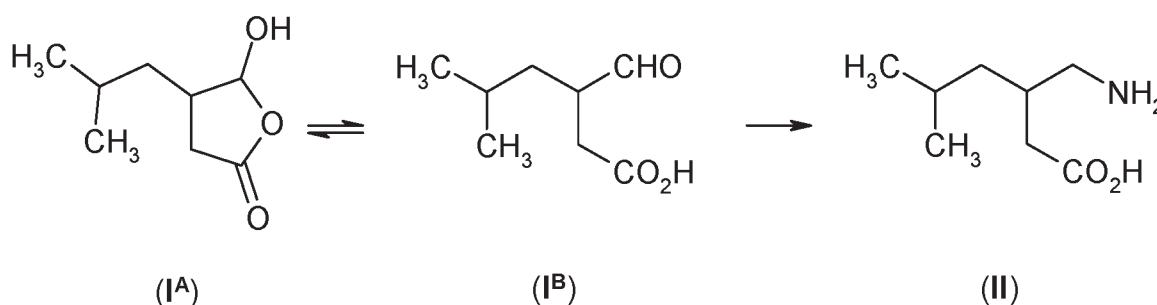
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$$100 \times (er-1) / (er +1)$$

where "er" is the ratio of the more abundant enantiomer to the less abundant enantiomer.

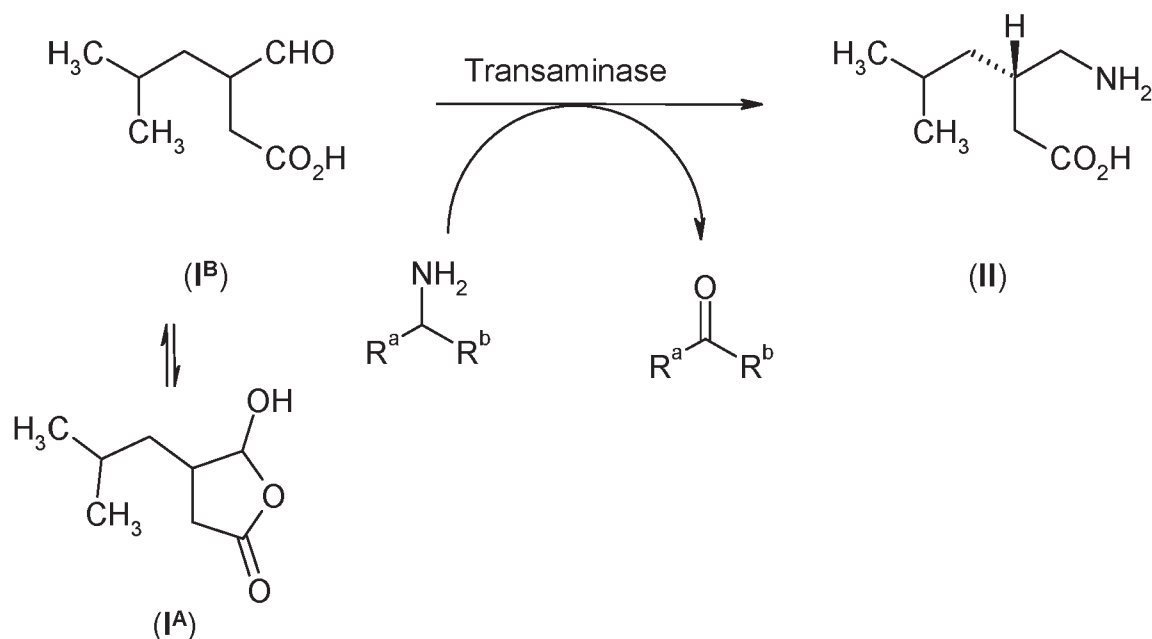
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5-Hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone (**I^A**) is a convenient intermediate in the manufacture of 3-aminomethyl-5-methylhexanoic acid (**II**). Treatment of racemic (**I^A**) with ammonia in the presence of a chemical reducing agent provides racemic 3-aminomethyl-5-methylhexanoic acid ((*R/S*)-**II**) (see WO2008/127646A2). The reaction is presumed to involve the ring-opened isomer (**I^B**).

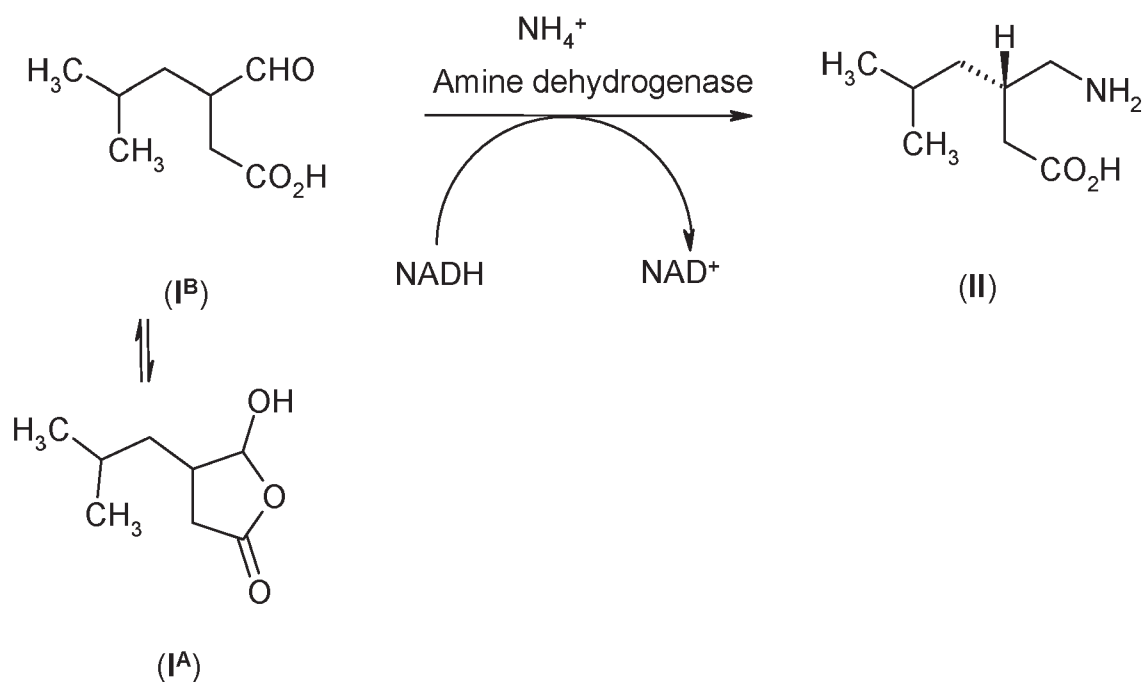


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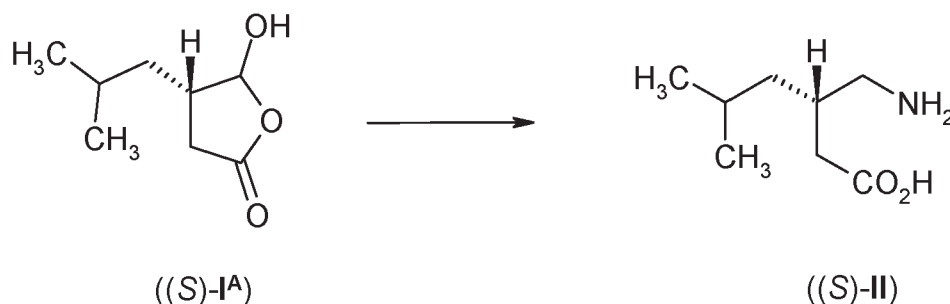
Reductive amination with an amine donor in the presence of a transaminase enzyme can provide enantiomerically enriched 3-aminomethyl-5-methylhexanoic acid. Suitable amine donors are primary amines such as mono-alkylamines, particularly isopropylamine, and α -amino acids.



The reaction of (I^A) with a suitable amine dehydrogenase/ imine reductase in the presence of ammonia can also be a suitable route to pregabalin. A co-factor such as NADH or NADPH may be needed in a stoichiometric amount, or a second
 5 oxidoreductase, such as formate dehydrogenase, may be included to recycle the co-factor.



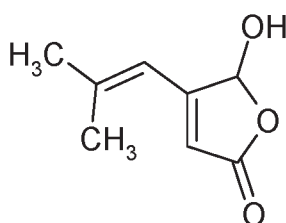
Where the dihydrofuranone (I^A) has a defined stereochemistry at the C-4 position, this
 10 stereochemistry may be preserved during the reductive amination reaction. Since Pregabalin has the (S)-stereochemistry it may be preferred to have this stereochemistry already present in the dihydrofuranone.



Alternatively, the dihydrofuranone (I^A) may be used as in racemic form. The desired stereoisomer of the product may then be obtained either by carrying out the reductive amination reaction under conditions that allow for the stereoselective formation of a single enantiomer, such as by carrying out the transformation in the presence of a transaminase enzyme, or by subjecting the product to a separate resolution step, such as by crystallization with a chiral acid or base.

- 10 The dihydrofuranone (I^A) may be conveniently prepared in racemic or enantiomerically enriched form using the methods set out below.

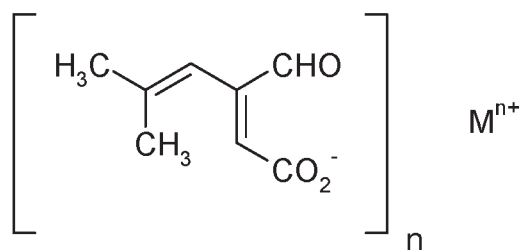
In a first method, the dihydrofuranone (I^A) is prepared by reduction of 5-hydroxy-4-(2-methyl-1-propenyl)-5H-2-furanone (VI^A).



(VI^A)

The reduction may conveniently be accomplished by hydrogenation in the presence of a suitable catalyst. Suitable catalysts include homogeneous and heterogeneous catalysts. The catalyst typically comprises a transition metal such as palladium, platinum, rhodium, ruthenium or nickel, or a salt or oxide thereof. Heterogeneous catalysts include finely divided metals and substrate-supported metals and metal oxides, where the substrate may be carbon, silica, alumina or any other suitable inert material. Homogeneous catalysts include phosphine ligand complexes of transition metals. When the phosphine ligand is chiral then the catalyst is chiral. When an achiral catalyst is used, then the product is the racemic dihydrofuranone (I^A). The use of a chiral catalyst may provide the dihydrofuranone (I^A) in an enantioselective manner.

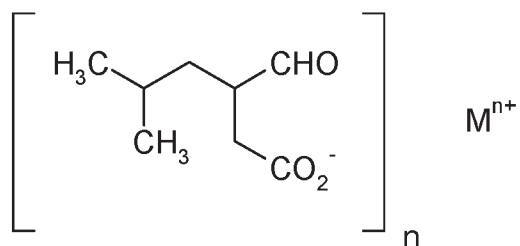
The selectivity of the hydrogenation reaction, and the overall yield, maybe improved when the reaction is carried out in an alkaline medium. Without being bound by theory, it is thought that in the presence of a base the furanone exists predominantly as the
 5 ring-opened salt (IX).



(IX)

Any suitable base may be used provided that it does not interfere with the hydrogenation process, such as by poisoning the catalyst. Examples of suitable bases include alkali (such as Li, Na, K and Rb) and alkaline earth metal (such as Ca and Mg)
 10 oxides, hydroxides, carbonates and bicarbonates. Other metal salts, such as zinc salts, may also be used. Alkali metal salts may be preferred due to their good solubility and/or low toxicity. Amine bases such as ammonia, and primary, secondary and tertiary amines may be used to prepare ammonium salts. Tetra-alkylammonium hydroxide may also be used, leading to the formation of tetra-alkylammonium salts.

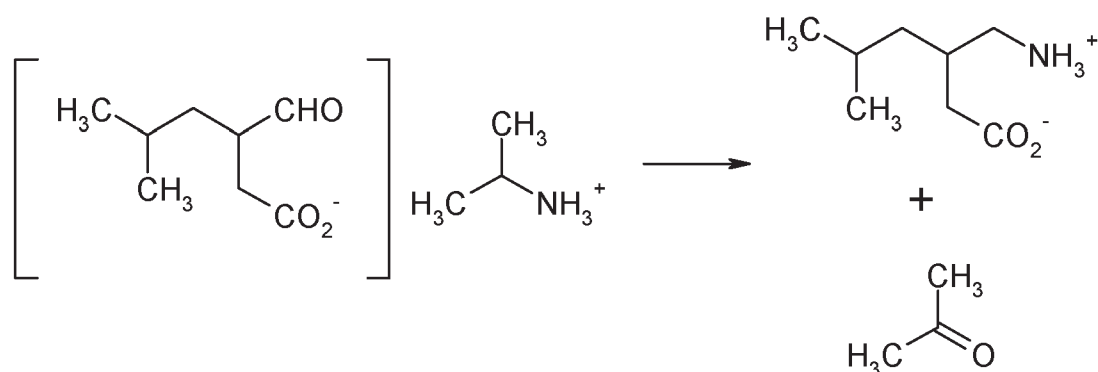
Hydrogenation of the salt of formula (IX) provides a salt of formula (X).



(X)

The dihydrofuranone (I^A) is recovered, after the hydrogenation reaction, by treatment of
 20 this salt with a suitable acid. Alternatively, the salt may be converted directly to 3-aminomethyl-5-methylhexanoic acid (II) by treatment with a transaminase or amine oxidase/imine reductase enzyme. In this case it may be preferable to use the ammonium salt ($\text{M}^+ = \text{NH}_4^+$) or a primary alkylammonium salt ($\text{M}^+ = \text{alkyl-NH}_3^+$) since the ammonium or alkylammonium ion provides the co-substrate for the enzyme. The

use of the isopropylammonium salt in combination with a transaminase enzyme is a preferred example.



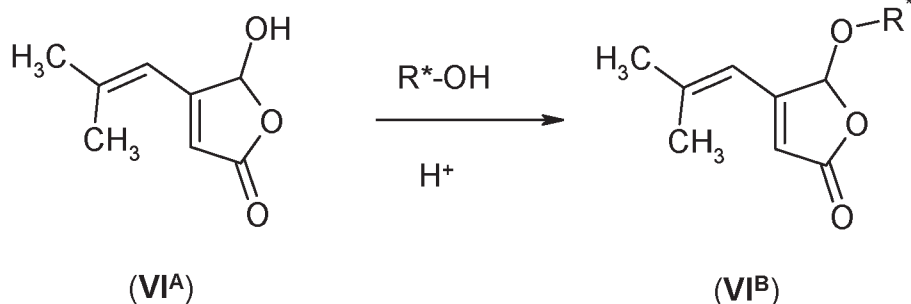
- 5 Furanones of formula (VI) wherein R is other than hydrogen may also be reduced by hydrogenation. Where R is an alkyl, haloalkyl, alkoxyalkyl alkenyl, cycloalkyl, cycloalkyl-alkyl, aryl or aryl-alkyl group, these furanones may be prepared from the compound of formula (VI^A) by reaction with an alcohol R-OH in the presence of an acid catalyst. Where R is R¹-C(O)- the furanones may be prepared from the compound of
- 10 formula (VI^A) by reaction with an acid anhydride (R¹-C(O))₂O or acid chloride R¹-C(O)-Cl, optionally in the presence of a base, for example a tertiary amine. Where R is R²-SO₂- the furanones may be prepared from the compound of formula (VI^A) by reaction with a sulfonyl chloride R²-SO₂-Cl, optionally in the presence of a base, for example a tertiary amine.

15 Following the hydrogenation step the dihydrofuranone of formula (I^A) can be generated from the reduction product by treatment with acid (where R is an alkyl, haloalkyl, alkoxyalkyl alkenyl, cycloalkyl, cycloalkyl-alkyl, aryl or aryl-alkyl group) or base (where R is R¹-C(O)- or R²-SO₂-).

20 The use of a chiral R group may provide a chiral hydrogenation product without the need for a chiral catalyst.

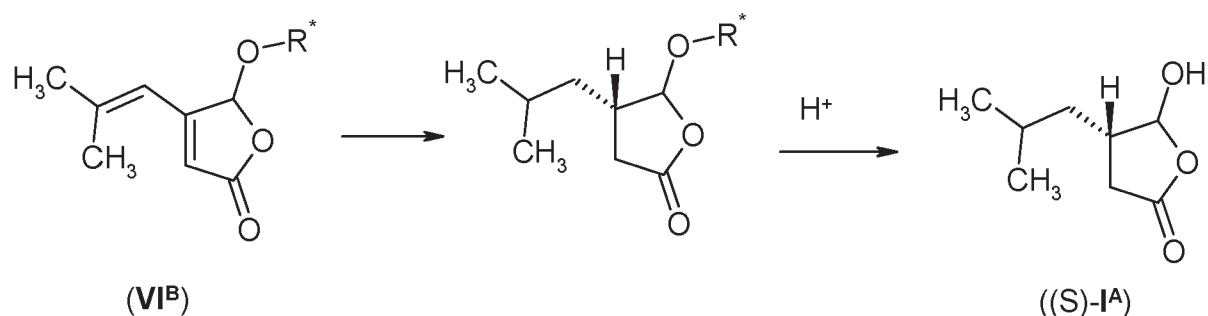
For example, the furanone (VI^A) is reacted with a chiral alcohol R*-OH to afford a chiral ether derivative (VI^B).

25

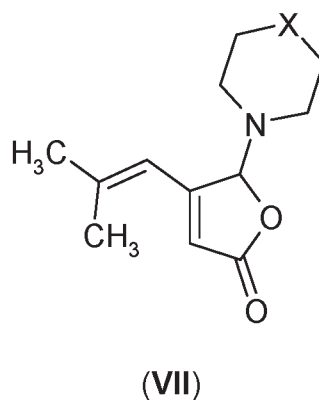


Suitable chiral alcohols may include α -aryl alcohols such as 1-phenylethanol and 1-naphthylethanol, as well as terpene alcohols such as menthol and borneol.

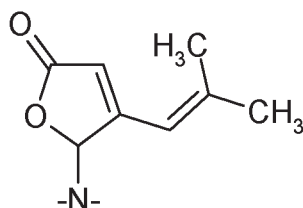
- 5 Hydrogenation of derivative (VI^B) may proceed in an enantioselective manner, and treatment of the resulting product with a suitable acid and in the presence of water then provides the dihydrofuranone (I^A) in chiral form.



The furanone (VI^A) may be prepared from a compound of formula (VII)



wherein -X- represents a single bond, -CH₂-, -O-, -NH-, -N((C₁-C₃)alkyl)-, -N(benzyl)-, or

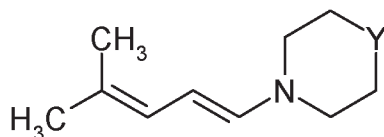


by treating the compound of formula (VII) with water in the presence of an acid catalyst.

- 15 Suitable acids include mineral acids such as sulphuric acid. Alternatively, the

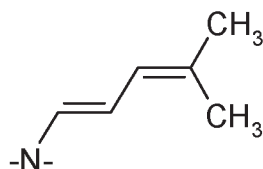
compound of formula (VII) can be treated with an alcohol R-OH to provide directly a compound of formula (VI) wherein R is an alkyl, haloalkyl, alkoxyalkyl alkenyl, cycloalkyl, cycloalkyl-alkyl, aryl or aryl-alkyl group.

- 5 The compounds of formula (VII) can be prepared by the reaction of a dienamine of formula (VIII).



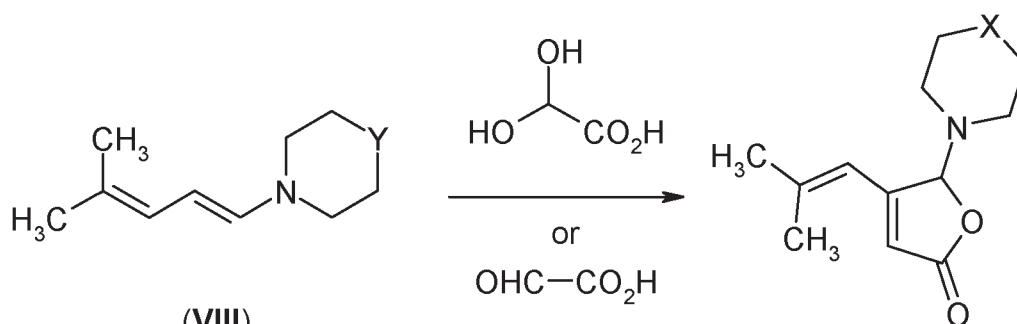
(VIII)

wherein -Y- represents a single bond, -CH₂-, -O-, -NH-, -N((C₁-C₃)alkyl)-, -N(benzyl)-, or



10

with glyoxylic acid or its hydrate.

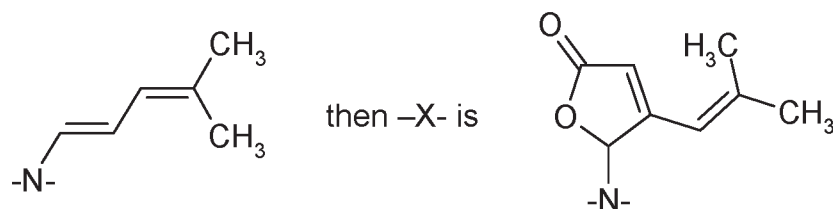


(VIII)

(VII)

It will be understood that -X- in formula (VII) corresponds to -Y- in the starting material of formula (VIII), except that when -Y- is

15

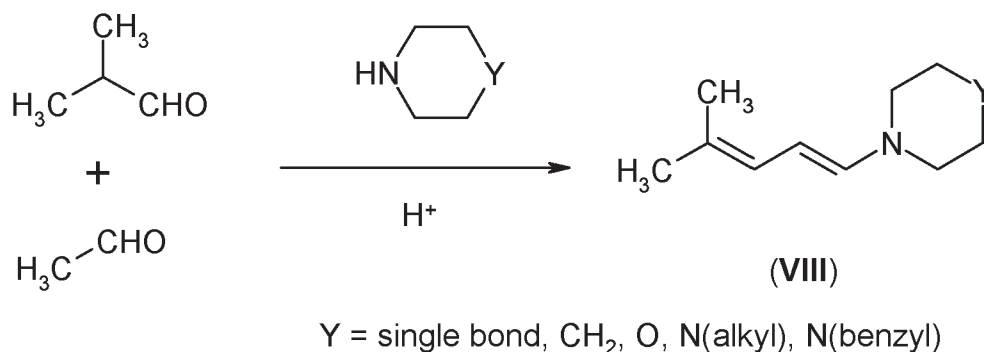


then -X- is

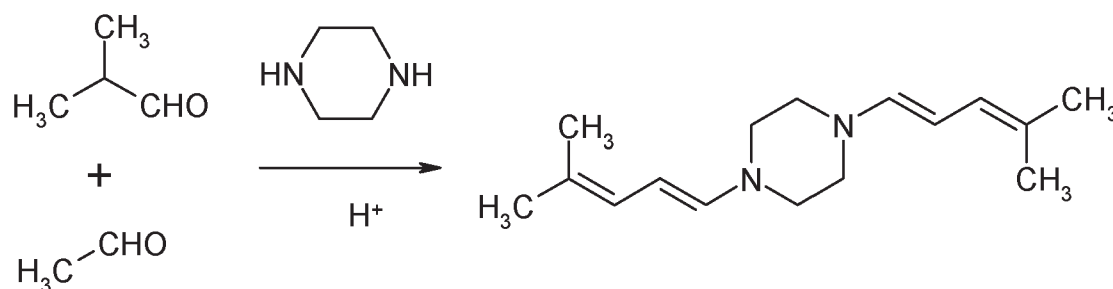
The compounds of formula (VIII) wherein -Y- represents a single bond or -CH₂- have been prepared by the reaction of 4-methyl-2-pentenal with pyrrolidine or piperidine

(Kienzle, F. et al., Helv. Chim. Acta 1985, 68(5), 1133-39). Other compounds of formula (VIII) may be prepared analogously.

Alternatively, condensation of isobutyraldehyde and acetaldehyde in the presence of a suitable amine such as pyrrolidine, piperidine or morpholine with catalytic acid in a solvent such as acetonitrile provides the dienamine derivatives (VIII).



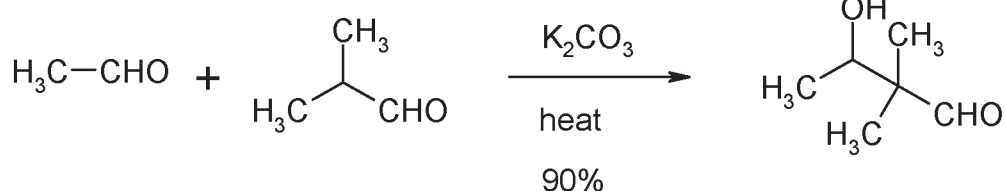
10 If piperazine is used as the amine then a *bis*-dienamine is obtained.



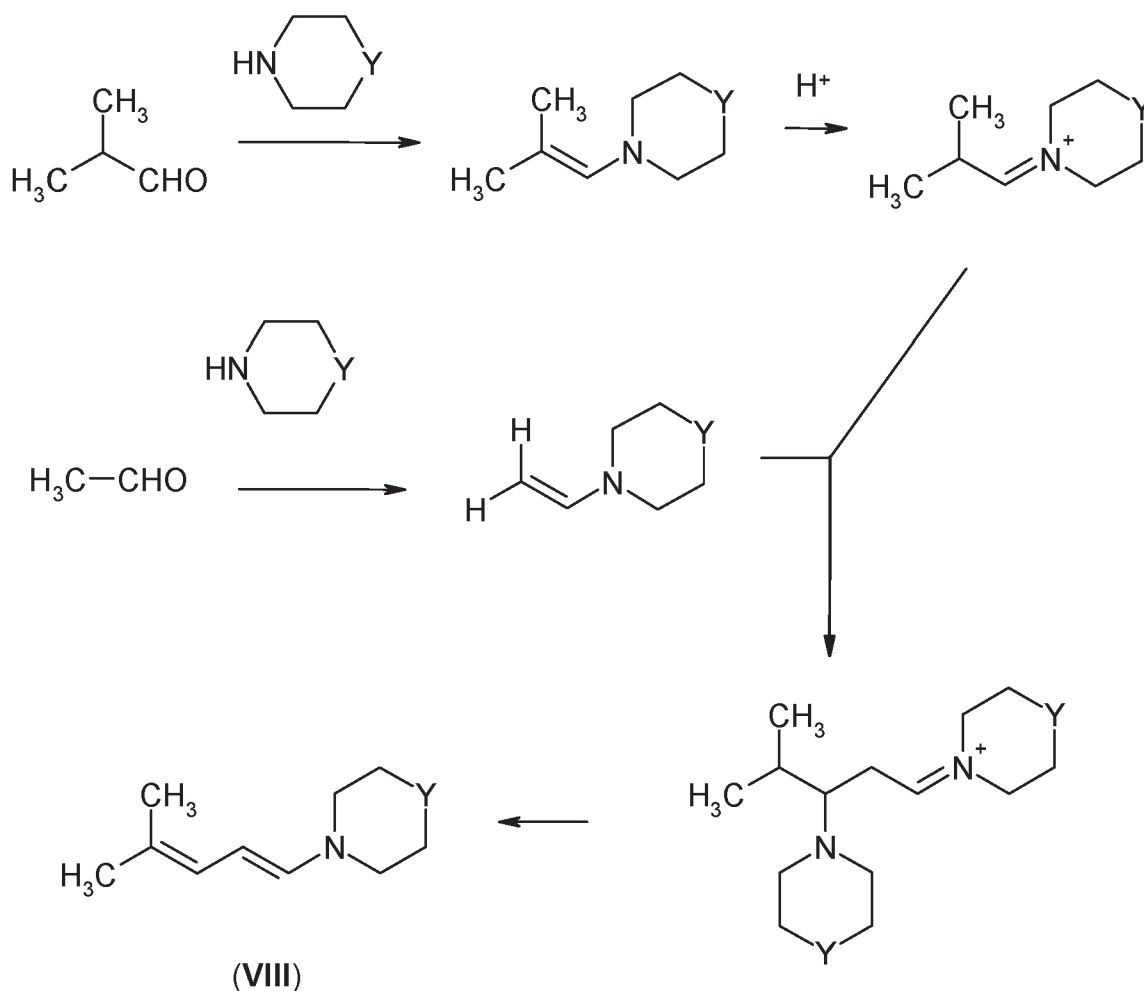
The compound of formula (VIII) wherein Y is NH may be obtained by using mono-protected piperazine as the amine, followed by a deprotection step.

Acyclic secondary amines such as diethylamine and di-isopropylamine may also be used, but cyclic secondary amines are preferred.

This mode of reaction of isobutyraldehyde and acetaldehyde differs from the direct base catalysed reaction (eg using potassium carbonate as base: UK patent GB834100) which only gives the adduct 2,2-dimethyl-3-hydroxybutanal where isobutyraldehyde acts as a nucleophile.



Without wishing to be bound by any particular theory, it is postulated that both the acetaldehyde and the isobutyraldehyde are initially converted to their enamine derivatives. In the presence of the acid catalyst, the more basic isobutyraldehyde enamine is converted to its iminium ion. This electrophilic species reacts preferentially with the less sterically hindered nucleophile, which is the acetaldehyde enamine – this ensures reaction the desired way around.



The method of the present invention is more economical and more amenable to scale-up than the literature methods of effecting this 'umpolung' of normal acetaldehyde reactivity, in which acetaldehyde is converted to an O-silylated enol derivative and coupled with the isobutyraldehyde under Mukiyama aldol conditions. In the present

invention, both coupling partners are activated simultaneously – electronic and steric effects directing the observed reactivity pattern. The other advantage is that the product dienamine is the desired ‘activated’ form of 4-methyl-2-pentenal for reaction with glyoxylic acid to form the desired 5-aminofuranones (VII).

5

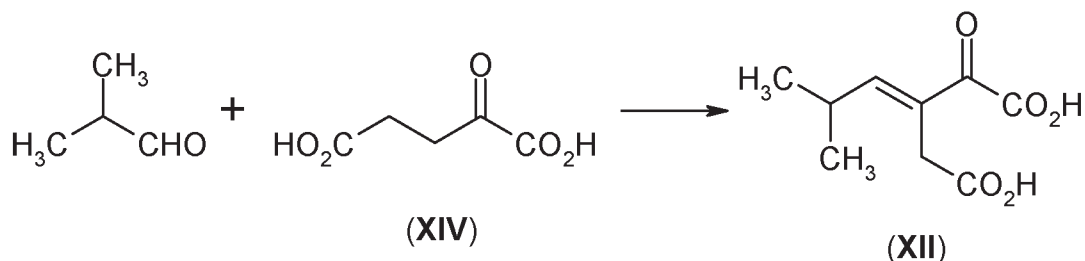
These dienamine derivatives of formula (VIII) may be isolated and purified, or alternatively they can be treated directly with glyoxylic acid (or its hydrate), which provides furanone derivative (VII) directly.

- 10 The furanone derivatives (VII) may be isolated and purified. Treatment with aqueous acid then provides furanone (VI^A).

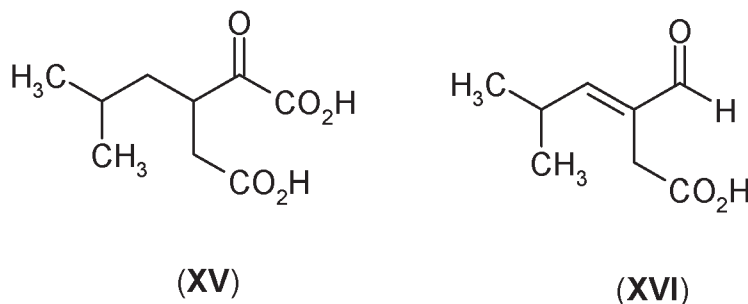
Overall, the transformations set out above provide a short route for the manufacture of pregabalin using inexpensive and safe starting materials.

15

In an alternative embodiment, the dihydrofuranone (I) is prepared from 3-isobutylidene-2-oxopentanedioic acid (XII), which is readily obtained by the condensation of isobutyraldehyde with 2-oxopentanedioic acid (α -ketoglutaric acid) (XIV).



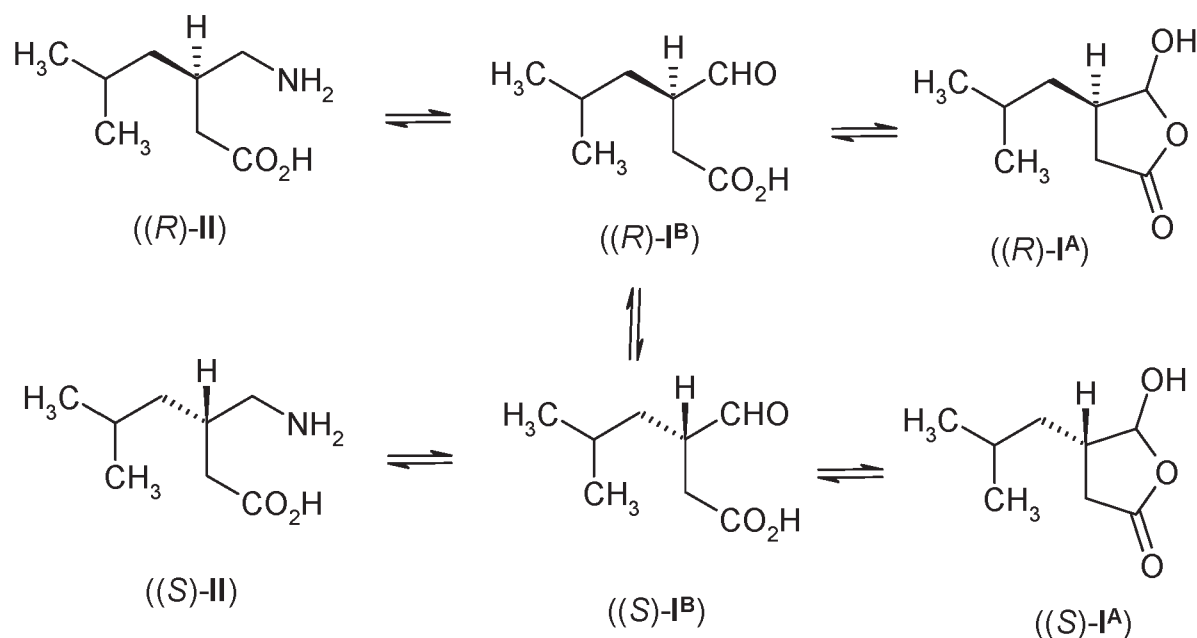
- 20 Conversion of diacid (XII) to dihydrofuranone (I) requires a decarboxylation step and a reduction step. These two process steps may be carried out separately, in which case either the decarboxylation step or the reduction step may be the first step, or the two processes may be carried out simultaneously. When the reduction step is carried out first, the intermediate (XV) will be produced. When the decarboxylation step is carried out first the intermediate (XVI) will be produced.



The reduction step may be carried out chemically, such as by hydrogenation, but is preferably achieved using an enzyme-mediated reduction, such as by treating with an enoate reductase enzyme. The decarboxylation step is preferably performed by treating the compound with a decarboxylase enzyme.

Where enzyme-mediated transformations are contemplated, the enzyme may be an isolated enzyme, including an enzyme immobilized on a carrier, it may be a partially isolated enzyme preparation such as a cell homogenate, or it may be a non-isolated enzyme, in which case a whole-cell preparation is used. Cells may include those which express the desired enzyme naturally, and cells that have been manipulated so as to express the desired enzyme.

The enzyme-mediated reductive amination of the compound of formula (I^A) is reversible, and so treatment of 3-aminomethyl-5-methylhexanoic acid (II) with a transaminase enzyme or an amine oxidase/imine reductase enzyme can lead to the formation of the dihydrofuranone (I^A). The ring-opened isomer of this is compound (I^B), which is epimerizable. In view of this, it is possible to convert ((R)-II) into ((S)-II), or to increase the optical purity of a mixture of ((R)-II) and ((S)-II) using such an enzyme.



Examples

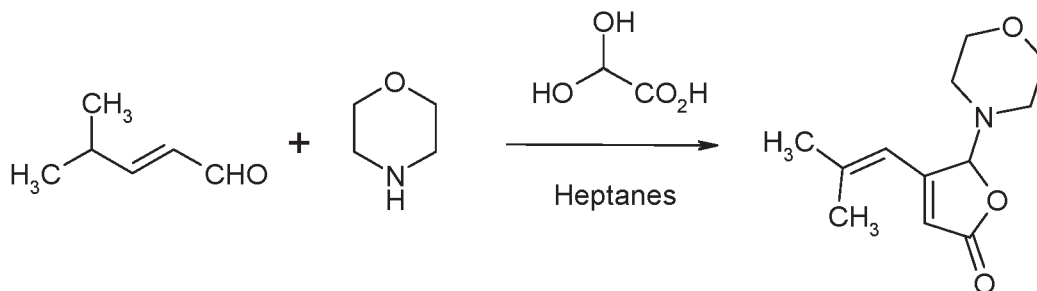
The invention is illustrated by the following non-limiting examples in which the following abbreviations and definitions are used:

5

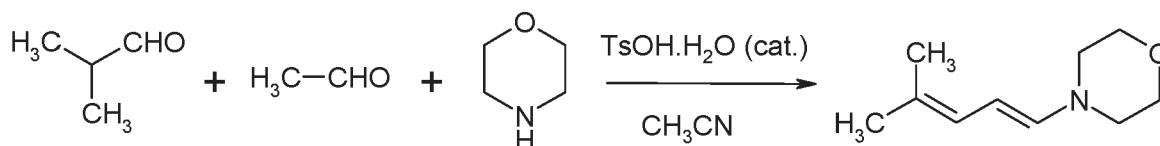
bp	Boiling point
CPME	Cyclopentyl methyl ether
d	Doublet
DIW	De-ionised water
dd	Doublet of doublets
eq or eq.	Equivalent
e.e. or ee	Enantiomeric excess
ES ⁺	Positive mode electrospray ionization
EtOAc	Ethyl Acetate
EtOH	Ethanol
GC	Gas chromatography
GC/MS	Gas chromatography / Mass spectroscopy
HOAc	Acetic acid
HPLC	High Performance Liquid Chromatography
Hr or h	Hour
¹ H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
L	Litre
LCMS	Liquid chromatography / Mass spectroscopy
m	Multiplet
mbar	Millibar
MeOH	Methanol
min	Minute
mL	Millilitre
mmol	Millimole
mol	Mole
Mp	Melting point
MTBE	Methyl tert-butyl Ether
NAD ⁺	Nicotinamide adenine dinucleotide (oxidised)
NADP ⁺	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PLP	Pyridoxal phosphate
ppm	Parts per million
pTsOH	Para-toluenesulfonic acid
q	Quartet
qNMR	Quantitative nuclear magnetic resonance spectroscopy
R _f	Retention factor
RT	Room temperature
s	Singlet
t	Triplet
ThDP	Thiamine diphosphate
TLC	Thin layer chromatography
TsOH	Toluenesulfonic acid (= pTsOH)
UPLC	Ultra Performance Liquid Chromatography
XRD	X-ray diffraction crystallography
δ	Chemical shift

Commercial chemicals were used as received unless stated otherwise. Thin layer chromatography was performed on pre-coated plastic plates (Merck silica 60F254), and visualised using UV light and KMnO₄ dip. Proton (¹H) and carbon (¹³C) NMR spectra
5 were recorded on a Varian INOVA 300 MHz spectrometer. Chemical shifts are quoted relative to tetramethylsilane and referenced to residual solvent peaks as appropriate. Unless otherwise indicated, chiral HPLC analysis was performed using an Agilent 1200 HPLC system and data was processed using the Chemstation software or with a Varian semiprep/analytical HPLC using Galaxie software.

Example 1**Preparation of 4-(2-methyl-1-propenyl)-5-morpholino-5H-2-furanone from 4-methyl-2-pentenal**

- 5 A 50% solution of glyoxylic acid in water (29.6g, 0.2mol) was added to a biphasic stirred mixture of morpholine (17.8g, 0.2mol) and heptanes (75mL), which had been pre-cooled to 0-10°C. The temperature was kept less than 10°C. The mixture was warmed to 20°C and 4-methyl-2-pentenal (19.6g, 0.2mol) was added. The mixture was stirred at 45°C for 20h. A large quantity of solid was formed. Water (100mL) was added at ambient
- 10 temperature and the mixture stirred for 2h. The mixture was extracted with cyclopentyl methyl ether (100mL) and the organic solution washed twice with water (100mL) and concentrated to leave 30.4g of crude product. This solid was purified by recrystallisation from methanol (100mL) to afford 17.7g (40%) of pure 4-(2-methyl-1-propenyl)-5-morpholino-5H-2-furanone.
- 15 GC/MS: m/z = 223
- ¹H NMR: δ 6.0 (s, 1H); 5.9 (s, 1H); 5.5 (s, 1H), 3.7 (d, 4H), 2.7 (d, 4H), 2.00 (s, 3H), 1.95 (s, 3H).

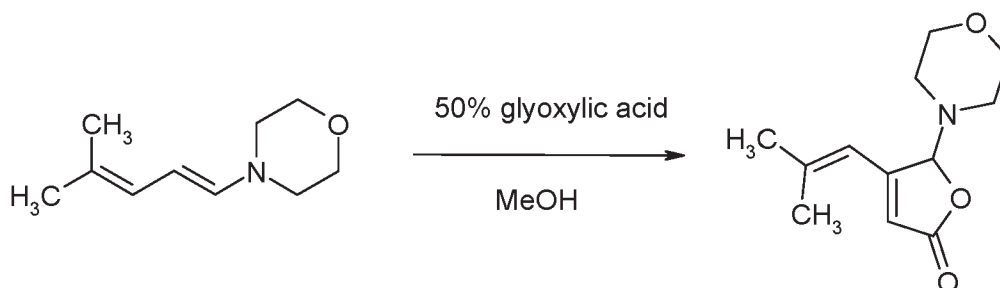
Example 2**Preparation of 4-(4-methyl-1,3-pentadien-1-yl)morpholine**

- Isobutyraldehyde (46.90g, 0.65mol, 1.43eq.) was stirred in acetonitrile (300mL). Morpholine (56.63g, 0.65mol, 1.43eq.) followed by pTsOH (8.63g, 0.1 equiv) were slowly added at room temperature to the isobutyraldehyde solution. A solution of
- 25 acetaldehyde (20g, 0.454mol) in acetonitrile (100mL) was added drop-wise over 1hr with internal temperature monitoring at 50°C. After complete addition the mixture was stirred for 30min at 50°C and then cooled to room temperature before evaporation of the solvent *in vacuo* to give an orange oil (123.3g). Assay of the crude by quantitative NMR

using benzyl benzoate as the internal standard was 40% which give 65% yield of the desired dienamine.

GC/MS: $m/z = 167$

^1H NMR: δ 6.01 (d, 1H); 5.69 (d, 1H); 5.31 (dd, 1H), 3.70 (m, 4H), 2.89 (m, 4H), 1.71 (s, 3H), 1.63 (s, 3H).

Example 3**Preparation of 4-(2-methyl-1-propenyl)-5-morpholino-5H-2-furanone from crude 4-(4-methyl-1,3-pentadien-1-yl)morpholine**

5

The crude 4-(4-methyl-1,3-pentadien-1-yl)morpholine dienamine of example 2 (123.3g, 40% assay) was dissolved in methanol (300mL) at room temperature. On complete dissolution, glyoxylic acid (50wt%, 60g, 1.1eq) was added and the resultant biphasic mixture stirred at 50°C for 18h. The reaction mixture was cooled to room temperature and the solvent removed by rotary evaporation. The residue was partitioned between ethyl acetate (200mL) and saturated sodium carbonate solution (200mL). The aqueous phase was extracted with ethyl acetate (100mL) and combined organics washed with brine and evaporated to a thick oil which solidified on standing (89.0g, 67% assay by qNMR, 60% yield).

10

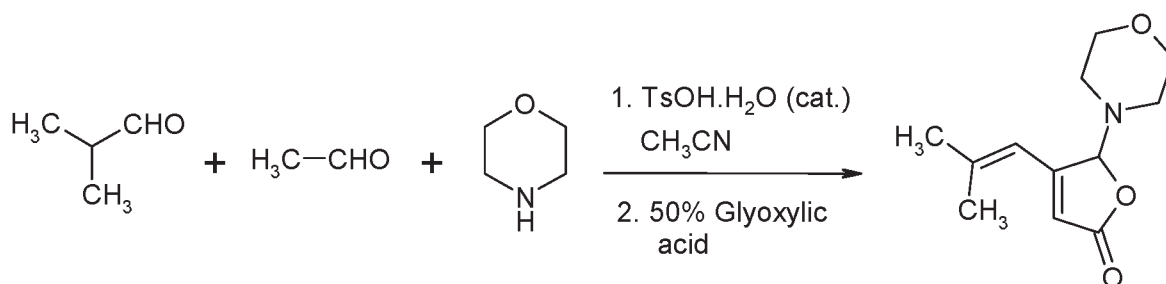
15

GC/MS: m/z = 223

NMR: as example 1.

Example 4**Preparation of 4-(2-methyl-1-propenyl)-5-morpholino-5H-2-furanone in one pot from isobutyraldehyde and acetaldehyde.**

20



Isobutyraldehyde (102.90g, 1.43mol) was stirred in acetonitrile (600mL). Morpholine (124.3g, 1.43mol) followed by pTsOH (19.0g, 0.1 equiv) were slowly added at room temperature to the isobutyraldehyde solution. A solution of acetaldehyde (44.05g, 1.0mol) in acetonitrile (150mL) was added drop-wise over 1hr with internal temperature monitoring at 50°C. After complete addition the mixture was stirred for 30min at 50°C

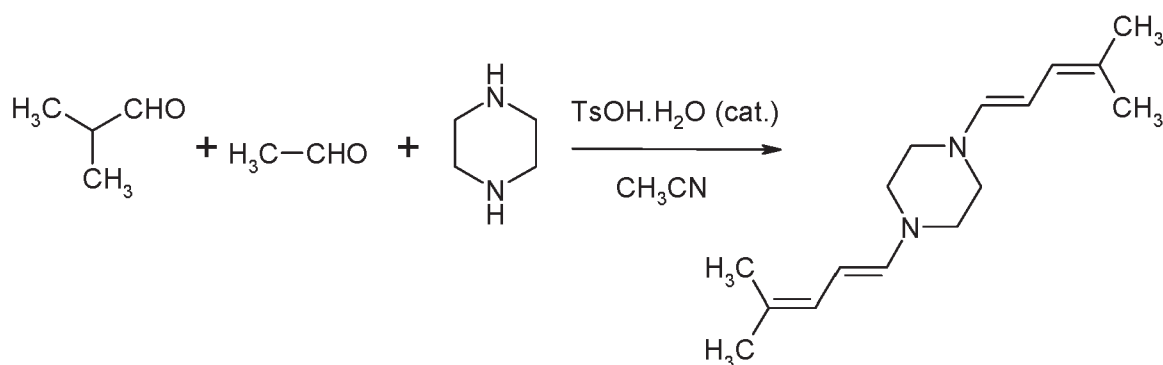
25

and then cooled to $<10^{\circ}\text{C}$. Glyoxylic acid (50wt%, 211.3g, 1.43mol) was added and the resultant biphasic mixture stirred at 50°C for 18h. The reaction mixture was cooled to room temperature and the solvent removed by rotary evaporation. The residue was partitioned between ethyl acetate (1L) and saturated sodium carbonate solution (1L).

- 5 The aqueous was extracted with ethyl acetate and combined organics washed with brine and evaporated to a thick oil which solidified on standing (166g). The crude product was triturated with MTBE at room temperature. The product is collected by filtration and washed with MTBE to give pure 4-(2-methyl-1-propenyl)-5-morpholino-5H-2-furanone (84g, 37% yield) identical to the material prepared in Example 1. Analysis of
10 the crude solid (166g) indicated ca 50% yield.

Example 5

Preparation of 1, 4-bis-(4-methyl-1,3-pentadien-1-yl)piperazine

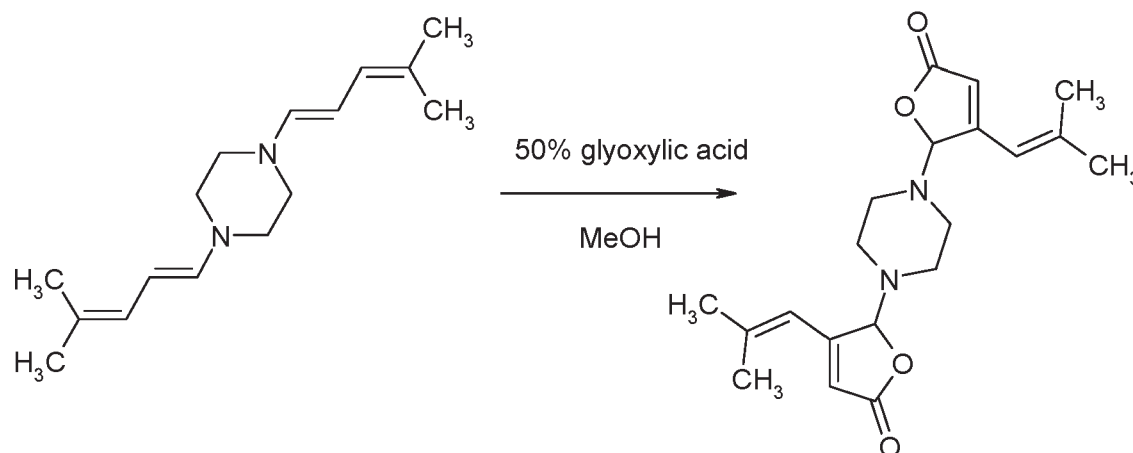


15

A solution of piperazine (43.0g, 0.5mol) and 4-toluenesulfonic acid monohydrate (4.4g, 0.023mol) in acetonitrile (600mL) was prepared. This solution was heated to 50°C and isobutyraldehyde (100mL, 1.10mol) added over 10 minutes. The solution went red-orange in colour and a transient white precipitate occurred. A solution of acetaldehyde
20 (30.8g, 0.70mol) in acetonitrile (30 mL) was then added via syringe pump over 3h at 50°C . A suspension was formed which was stirred at 50°C for 0.5h. The solvent was removed and the residual solid isolated from methanol (500mL) at -5°C . It was filtered and washed with chilled methanol and dried to afford 52.9g (60%) of 1,4-bis-(4-methyl-1,3-pentadien-1-yl)piperazine.

25 GC/MS: $m/z = 246$.

^1H NMR: δ 6.00 (d, 2H); 5.70 (d, 2H); 5.28 (dd, 2H); 2.92 (s, 8H); 1.73 (s, 6H); 1.68 (s, 6H). ^{13}C NMR (CDCl_3): 140.6 (C), 126.3 (CH), 123.4 (CH), 100.2 (CH), 48.2 (CH_2), 25.8 (CH_3), 18.1 (CH_3).

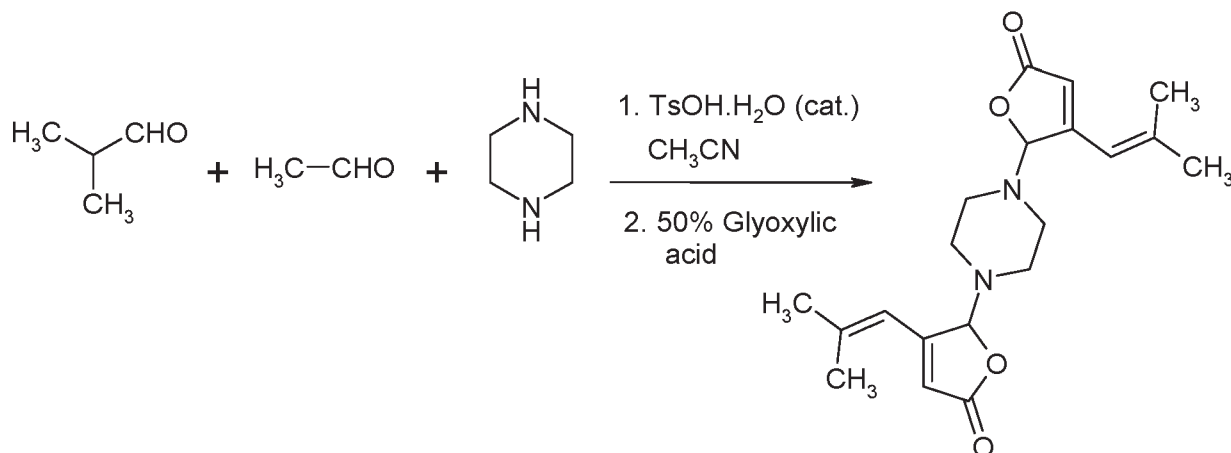
Example 6**Preparation of 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) from 1,4-bis-(4-methyl-1,3-pentadien-1-yl)piperazine.**

- 5 1,4-bis-(4-Methyl-1,3-pentadien-1-yl)piperazine (37.3g, 0.151mol; see Example 5) was charged to methanol (300mL). The temperature was adjusted to 34°C and a 50% solution of glyoxylic acid in water (44.9g, 0.302 mol) was added rapidly (over 5 minutes). The resulting suspension was stirred at 45°C for 15h; cooled to 0-10°C for 2h and filtered. The solid was washed with methanol (100mL) and dried to afford pure 5,5'-
- 10 (piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) (32.3g, 60%). An extra 5% could be obtained from the mother-liquor by concentration.

^1H NMR: δ 5.96 (d, 2H), 5.87 (d, 2H), 5.56 (m, 2H), 2.71 (s, 8H), 2.07-1.90 (m, 12H). ^{13}C NMR (CDCl_3): 172.3 (C), 159.2 (C), 150.9 (C), 116.6 (CH), 115.4 (CH), 99.2 (CH), 46.7 (CH_2), 28.2 (CH_3), 21.4 (CH_3).

15

This reaction can also be conducted in isopropanol, acetonitrile-water, toluene-water or in heptane water with similar yields.

Example 7**Preparation of 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) in one pot from isobutyraldehyde and acetaldehyde.**

- 5 Tonic acid (6.5g, 0.03mol) was charged to a solution of piperazine (59g, 0.69 mol) in acetonitrile (240mL). The reaction was heated to 50°C and agitated until dissolution of solids was observed, before addition of isobutyraldehyde (138mL, 1.51mol). The reaction was held at 50°C before a solution of acetaldehyde (60mL; 1.07mol) in acetonitrile (30mL) was charged to the vessel over 3hrs via syringe pump. On complete
- 10 addition, the reaction was stirred for a further 30 minutes before a 50% w/w solution of glyoxylic acid in water (148g, 1.0 mol) was added over 5min to the reaction mixture followed by water (50ml). The reaction was then heated to 70°C for 2h, then to 50°C overnight. The reaction was then cooled to 5°C and held for 30 minutes. 5,5'-(Piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) precipitated and
- 15 was isolated by filtration and washed with acetonitrile (2 x 100mL) to give the desired product (126 g, 93.5% assay, 66% yield from acetaldehyde).

Example 8**Solvent free preparation of 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one)**

- 20 Isobutyraldehyde (79g, 100mL, 1.1mol) was charged to a 3-necked round bottom flask and argon was flushed through the system. Piperazine (43g, 0.5 mol) and TsOH-H₂O (4.4g; 0.023 mol) were divided into 4 equal portions containing piperazine (10.75 g) and TsOH-H₂O (1.1g). Addition of the first portion of piperazine (10.75g) resulted in
- 25 exotherm from 24°C to 35°C. This was followed by the first portion of TsOH (1.1g). The reaction was agitated until all the piperazine had dissolved (t=35°C), after which the second portion of piperazine (10.75g) was added, followed by TsOH (1.1g) (t=41°C).

Stirring was continued until all of piperazine had dissolved ($t=41^{\circ}\text{C}$), then the third portion of piperazine (10.75g) was added, followed by TsOH (1.1g). After the 3rd charge a clear solution was generated and then the fourth portion of piperazine (10.75g) & TsOH-H₂O were added, followed by TsOH (1.1g) ($t=52^{\circ}\text{C}$). On complete addition the reaction was stirred at 50°C for 30 min.

Another flask (50mL) was charged with acetaldehyde (30.8g, 39mL, 0.7mol) and placed in an ice-water bath ($0-2^{\circ}\text{C}$). The acetaldehyde flask was connected to the 3-necked flask by cannula via septa. A stream of argon was then passed through the acetaldehyde flask at the rate which ensures that the addition of the acetaldehyde was complete within 3 h. After all acetaldehyde was added, the reaction was stirred for 30 min at 50°C , then cooled to 40°C and 50% glyoxylic acid (104g, 78mL) was added dropwise over 45 min at a rate to keep temperature below 50°C . Water (100mL) was then added and the reaction was heated at 70°C for 5 h. The reaction was cooled to 4°C in an ice-water bath. The precipitated product was filtered and filter cake was washed with cold water (100 mL). After drying in vacuo at 50°C 88.9g (71%) of desired product was obtained. Precipitate contains 78.5% of the title compound (HPLC assay).

Example 9

Alternative preparation of 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) with simultaneous addition of both aldehydes

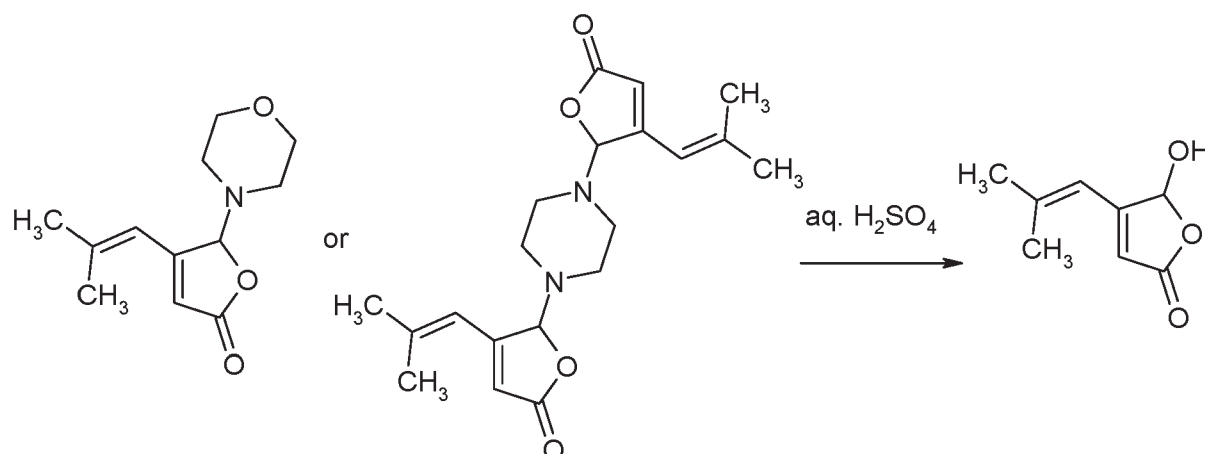
Piperazine (236.6g) was charged to a clean 3L dry vessel fitted with thermometer, addition funnel (100mL) and reflux condenser. pTsOH (25.3g) was charged to vessel followed by acetonitrile (550mL). The agitator was started and the vessel was inerted with nitrogen. Isobutyraldehyde (150mL, 27% of the total charge) was charged to the agitated piperazine / pTsOH slurry and a temperature rise to ca 43°C was observed. The white suspension was then heated to 50°C ($\pm 5^{\circ}\text{C}$). A pre-mixed chilled solution of isobutyraldehyde (400mL, 73% of total charge) and chilled acetaldehyde (260mL) were charged to a clean, dry 1L vessel and this mixture was maintained in an ice bath. The isobutyraldehyde / acetaldehyde mixture was charged to the contents of the 3L vessel in 100mL aliquots over 5-6h at 50°C . The contents of the reaction flask changed from a white suspension to a wine red solution and finally an orange suspension during the addition of the acetaldehyde. After complete addition the suspension was agitated at 50°C for ca. 0.5 to 1.0h.

Aqueous glyoxylic acid (50% wt/wt) solution was charged to the suspension over 10-15 min and the temperature rose to ca 75°C . 5,5'-(Piperazine-1,4-diyl)bis(4-(2-methylprop-

1-en-1-yl)furan-2(5H)-one) was observed to crystallise from solution. The suspension was stirred at 70°C (+/- 5°C) for 6h, then cooled with stirring to ambient. The batch was then cooled to -5 to 0°C for and held for 3-4h before the suspension was filtered and the cake washed with chilled methanol (1 x 500mL) then 2 x 500mL of methanol at ambient temperature. The washed product was dried in a vacuum oven at 40-50°C to constant weight to afford 474g of 98% pure 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) (70%).

Example 10

Preparation of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one from 4-(2-methyl-1-propenyl)-5-morpholino-2(5H)-furanone or 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one).



A 10% w/w aqueous sulphuric acid solution (110g) was charged to 4-(2-methyl-1-propenyl)-5-morpholino-2(5H)-furanone (20.0g, 0.896mol) and the mixture was stirred at reflux for 4h or until TLC (100:3 CPME: HOAc) indicated reaction completion. The mixture remained a suspension at all times. It was then cooled to 5°C, held for 2h, and filtered. The white solid was washed with water and dried to afford 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one (12.9g, 93%).

Alternatively, a 10% w/w aqueous solution of sulphuric acid (412g) was charged to a vessel containing 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) (60g, 0.167mol). The contents were agitated and then heated to reflux. The reaction was held at reflux until the starting material was consumed, which was determined by dissolution. On completion of reaction the batch was cooled to 35°C at which point the target compound began to crystallise. The slurry was further cooled to 0-5°C and then transferred to a filter. The product was filtered, washed with water (2 x

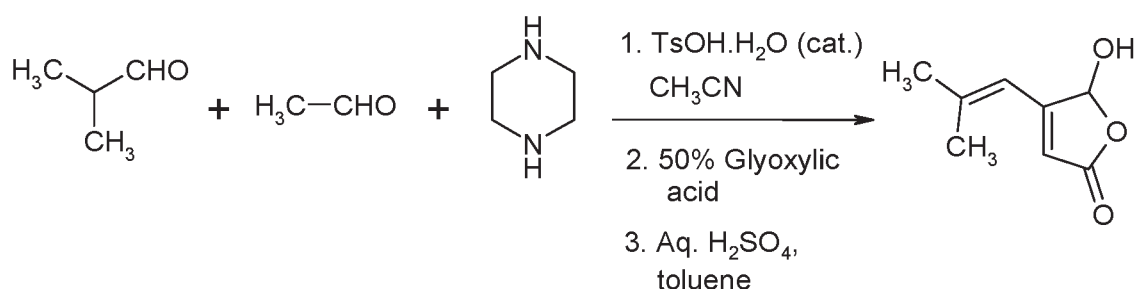
100mL) and then dried under vacuo at <50°C, to give 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one (42.0g, 81%).

GC/MS: m/z = 154

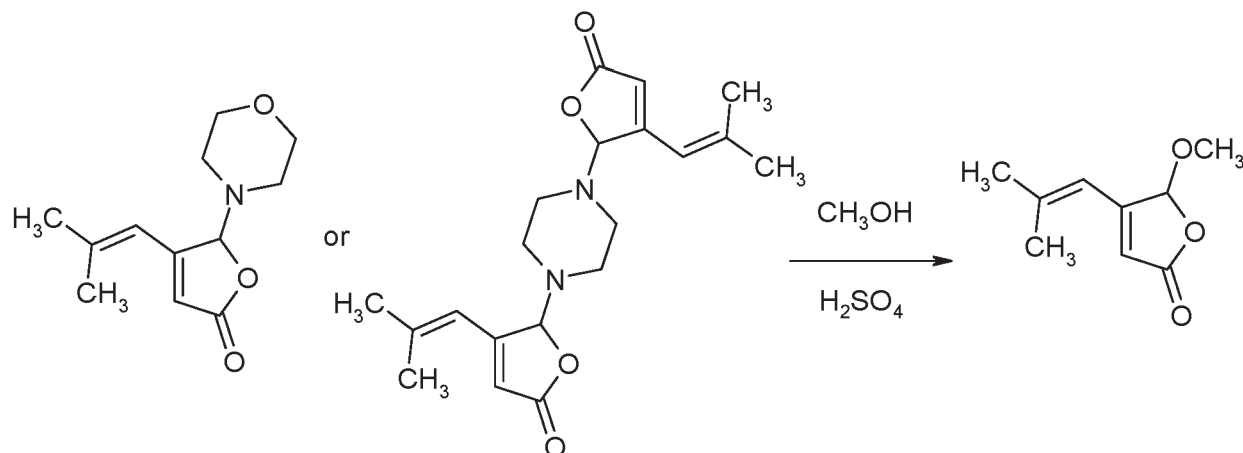
¹H NMR (CDCl₃): δ 6.10 (s, 1H); 5.92 (s, 1H); 5.88 (s, 1H); 5.35 (bs, 1H, O-H); 1.98 (s, 3H); 1.93 (s, 3H). ¹³C NMR (CDCl₃): 172.8 (C), 161.4 (C), 152.3 (C), 115.3 (CH), 114.9 (CH), 99.5 (CH), 28.2 (CH₃), 21.4 (CH₃).

Example 11

Preparation of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one in one pot from isobutyraldehyde and acetaldehyde.



A mixture of piperazine (43.0g, 0.5 mol) and isobutyraldehyde (200mL) were refluxed under Dean-Stark until the theoretical amount of water (18mL) collected. The excess isobutyraldehyde was distilled off to leave a crystalline mass of 1,4 bis(2-methylpropen-1-yl)piperazine. This was dissolved in acetonitrile (1.2L) and extra isobutyraldehyde (100 mL, 1.0mol) added. Extra piperazine (4.4g) was added followed by p-toluenesulfonic acid (4.4g, 23.2mmol). The temperature was adjusted to 40°C and a solution of acetaldehyde (44g, 1.0mol) in acetonitrile (40mL) was added over 4h. The reaction mixture was stirred at 40°C for 1h and at ambient temperature for 4h. The acetonitrile was distilled off and the residue suspended in toluene (400mL). A mixture of 50% glyoxylic acid (148g) and water (150mL) was added over 0.5h. The mixture was then stirred at 45°C for 15h. There was a solid precipitate. Dilute sulphuric acid (10%, 1L) was added and the mixture refluxed for 3h. A biphasic mixture resulted. The toluene phase was separated. It contained ca 50% yield (based on acetaldehyde) of 5-hydroxy-4-(2-methylpropen-1-yl)-2-furanone by analysis

Example 12**Preparation of 5-methoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one**

- 5 4-(2-Methyl-1-propenyl)-5-morpholino-2(5H)-furanone (7.70g, 34.5mmol) was stirred in MeOH (50mL) with H₂SO₄ (4mL, 2.2eq.) at reflux for 3h until disappearance of the starting material by GC. The reaction mixture was cooled to room temperature. The solvent was then evaporated *in vacuo* and the residue was diluted with EtOAc (50mL). The organic phase was washed with H₂O (3x 50mL). Solvent was then evaporated *in*
- 10 *vacuo* to give 5-methoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one as an orange oil (5.50g, 95%) which can be used without purification.

- Alternatively, 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) (130g, 363mmol) and MeOH (690mL) were charged in 2L round bottom flask with
- 15 mechanical agitation to form a suspension. Concentrated H₂SO₄ (43mL, 762mmol) was added dropwise with stirring at 20-25°C – a slight exotherm was observed, and nature of the solids present changed from dispersed and easily mixed to a thicker slurry. The reaction mixture was refluxed for 5h. After 0.5h complete dissolution (orange liquid) was observed. After 5h LCMS showed ~99% of the desired product. The mixture was
- 20 cooled to ambient and left for crystallization of piperazine sulfate. The sediments were filtered, and filter cake was washed with ice cold MeOH (400mL). The filtrate was concentrated *in vacuo* (40°C bath temperature), and the residue was partitioned between water (50mL) and MTBE (300mL). Water layer was separated and additionally extracted (2x300mL of MTBE). Combined organic extracts were washed with saturated
- 25 aq. NaHCO₃ (200mL). MTBE layer was dried over Na₂SO₄ with stirring for 1.5h, filtered and evaporated to give 5-methoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one as an orange oil (120g, 98%).

Distilled at 0.2-0.3 mbar vacuum with bp 100-105°C. GC/MS: m/z = 168; ¹H NMR (400 MHz, CDCl₃) δ 5.59 (1H, s) 5.88-5.86 (1H, m) 5.75 (1H, d, J=0.6) 3.53 (3H, s) 2.01-2.00 (3H, m) 1.97-1.96 (3H, m). ¹³C NMR (CDCl₃): 171.4 (C), 159.2 (C), 151.9 (C), 115.8 (CH), 115.2 (CH), 104.4 (CH), 56.1 (CH₃), 28.2 (CH₃), 21.4 (CH₃).

The following compounds were prepared from 4-(2-methyl-1-propenyl)-5-morpholino-2(5*H*)-furanone according to the first of the above methods by replacing methanol with 3-methylbutanol (isoamyl alcohol), *n*-pentanol (amyl alcohol) or *n*-butanol:

Example 12A

5-(3-Methylbutoxy)-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one

Distilled at 0.4 mbar vacuum with bp 139-140°C; GC/MS: m/z = 224; ¹H-NMR (400 MHz, CDCl₃) δ 5.94 (1H, s) 5.87-5.84 (1H, m) 5.80 (1H, s) 3.86-3.80 (1H, m) 3.70-3.64 (1H, m) 2.00 (3H, s) 1.96 (3H, s) 1.77-1.66 (1H, m) 1.57-1.50 (2H, m) 0.93-0.91 (3H, m) 0.91-0.89 (3H, m)

Example 12B

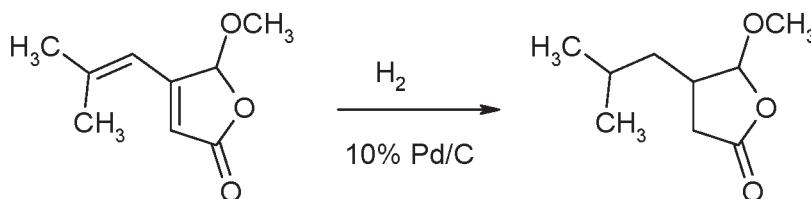
4-(2-Methylprop-1-en-1-yl)-5-pentyloxyfuran-2(5H)-one

Distilled at 0.08 mbar vacuum with bp 123-134°C; GC/MS: m/z = 224; ¹H-NMR (400 MHz, CDCl₃) δ 5.94 (s, 1H); 5.86 (s, 1H); 5.80 (s, 1H); 3.84-3.75 (m, 1H); 3.68-3.59 (m, 1H); 2.01 (s, 3H); 1.95 (s, 3H); 1.69-1.59 (m, 2H); 1.38-1.29 (m, 4H); 0.95-0.85 (m, 3H)

Example 12C

5-Butoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one

Distilled at 0.05 mbar vacuum with bp 136-146°C; GC/MS: m/z = 210; ¹H-NMR (400 MHz, CDCl₃) δ 5.94 (s, 1H); 5.86 (s, 1H); 5.80 (s, 1H); 3.85-3.76 (m, 1H); 3.69-3.60 (m, 1H); 2.00 (s, 3H); 1.95 (s, 3H); 1.68-1.58 (m, 2H); 1.45-1.32 (m, 2H); 0.93 (t, 3H, J=7.4 Hz)

Example 13**Preparation of 5-methoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one**

- 5 A solution of 5-methoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one (100 g, see example 12) in MTBE (700mL), 5g (5 wt%) of 10% Pd/C was charged to a hydrogenation vessel. 1atm of hydrogen gas was introduced and pressure was kept constant during the reaction. After 7h at 20°C, the reaction was filtered through a celite pad (ø60mm, H=30mm) and rinsed with MTBE (3x50mL). The filtrate was washed with 1M NaHCO₃
- 10 solution (200mL), water, brine and dried on Na₂SO₄. After solvent removal 5-methoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one was obtained as colourless liquid (98g, 96%).

GC/MS: m/z = 172

- ¹H NMR (400 MHz, CDCl₃) δ 5.25 (d, *J* = 5.0 Hz, 0.53 H, major isomer), 5.04 (d, *J* = 2.4 Hz, 0.26H, minor isomer), 3.48 (s, 0.92H, minor isomer), 3.46 (s, 1.66H, major isomer), 2.78 (dd, *J* = 17.7, 8.6 Hz, 0.33H), 2.59 – 2.42 (m, 1.27H, major isomer), 2.42-2.34 (m, 0.33H, minor isomer), 2.29 (dd, *J* = 16.7, 11.8 Hz, 0.63H, major isomer), 2.15 (dd, *J* = 17.7, 4.6 Hz, 0.33H, minor isomer), 1.67 – 1.31 (m, 2.63H both isomers), 1.28 – 1.19 (m, 0.33H, minor isomer), 0.95 – 0.86 (m, 6H both isomers). ¹³C NMR (CDCl₃): 177.7 (C, major isomer), 176.04 (C, minor isomer), 110.1 (CH, minor isomer), 106.1 (CH, major isomer), 57.0 (CH₃, minor isomer), 56.6 (CH₃, major isomer), 41.2 (CH₂, minor isomer), 39.1 (CH₂, minor isomer), 38.3 (CH₂, major isomer), 37.1 (CH₂, major isomer), 33.9 (CH, minor isomer), 32.8 (CH, major isomer), 26.0 (CH, major isomer), 25.8 (CH, minor isomer), 23.0 (CH₃, major isomer), 22.9 (minor isomer), 22.7 (major isomer), 22.6 (minor isomer).

Using the same general method and starting from the compounds of examples 12A, 12B and 12C respectively, the following compounds were also obtained:

Example 13A**5-(3-Methylbutoxy)-4-(2-methylpropyl)-dihydrofuran-2(3H)-one**

GC/MS: m/z = 228; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.35 (0.86H, d, $J=5.0$ Hz) 5.13 (0.14H, d, $J=2.7$ Hz) 3.87-3.79 (m, 1H) 3.57-3.45 (m, 1H) 2.58-2.37 (2H, m) 2.35-2.27 (0.79H, m) 2.20-2.11 (0.21H, m) 1.74-1.62 (1H, m) 1.61-1.44 (4H, m) 1.42-1.31 (1H, m) 0.95-0.86 (12H, m)

5

Example 13B

4-(2-Methylpropyl)-5-pentyloxydihydrofuran-2(3H)-one

GC/MS: m/z = 228; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.35 (0.77H, d, $J=5.0$ Hz) 5.13 (0.23H, d, $J=2.6$ Hz,) 3.83-3.75 (1H, m) 3.55-3.40 (1H, m) 2.59-2.37 (2H, m) 2.32 (0.73H, dd, $J=16.6, 11.8$ Hz) 2.15 (0.27H, dd, $J=17.7, 5.0$ Hz) 1.67-1.45 (4H, m) 1.41-1.25 (5H, m) 0.96-0.86 (9H, m)

10

Example 13C

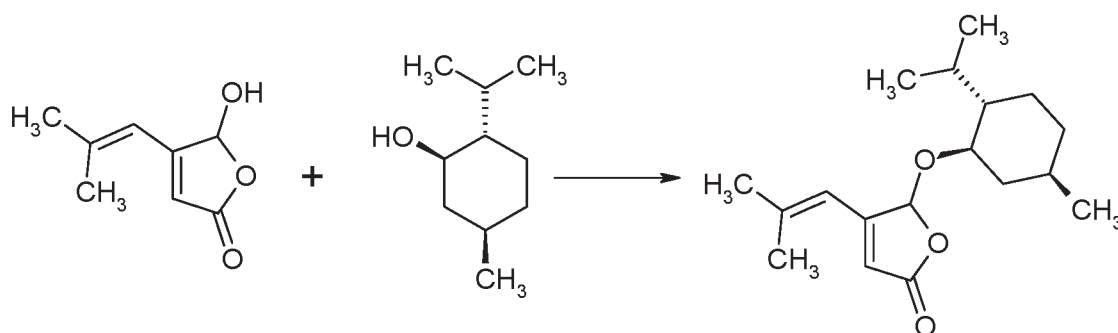
5-Butoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one

GC/MS: m/z = 214; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.35 (0.8H, d, $J=5.0$ Hz) 5.13 (0.2H, d, $J=2.6$ Hz,) 3.85-3.75 (1H, m) 3.56-3.41 (1H, m) 2.59-2.38 (2H, m) 2.31 (0.76H, dd, $J=16.5, 11.7$ Hz) 2.15 (0.24H, dd, $J=17.6, 5.0$ Hz) 1.66-1.45 (4H, m) 1.43-1.30 (3H, m) 0.95-0.87 (9H, m)

15

Example 14

Preparation and hydrogenation of 5-(L-Menthylloxy)-4-(2-methyl-1-propenyl)-2(5H)-furanone



A mixture of 5-hydroxy-4-(2-methyl-1-propenyl)-2-furanone (59.5g, 0.386 mol), L-menthol (89.5g, 1.5 equivalents) and methanesulfonic acid (1.5g) was stirred at 70-80°C under vacuum (to remove water) for 100h. The liquid mass was poured (hot) into acetonitrile (450mL) and the title compound isolated by cooling, filtration and washing. The yield was 76.2g (67%). Suitable crystals for XRD were grown by slow evaporation of an acetone solution. The configuration at the acetal carbon was (*R*).

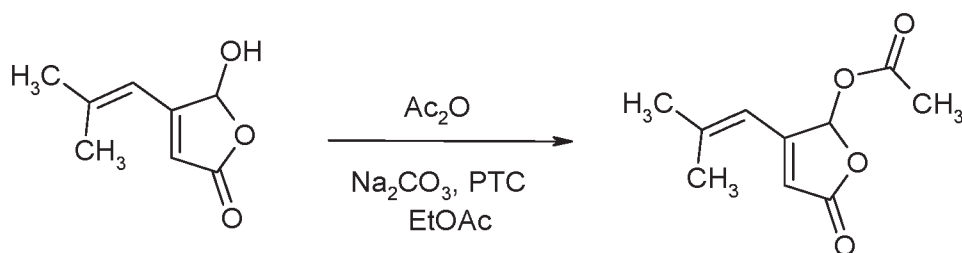
25

Hydrogenation of 5-(L-menthyloxy)-4-(2-methyl-1-propenyl)-2-furanone in ethyl acetate using the conditions of Example 13, gave the saturated derivative in quantitative yield. By ^1H NMR, the compound was a 1:1 mixture of diastereomers.

5

Example 15

Preparation of 5-acetoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one

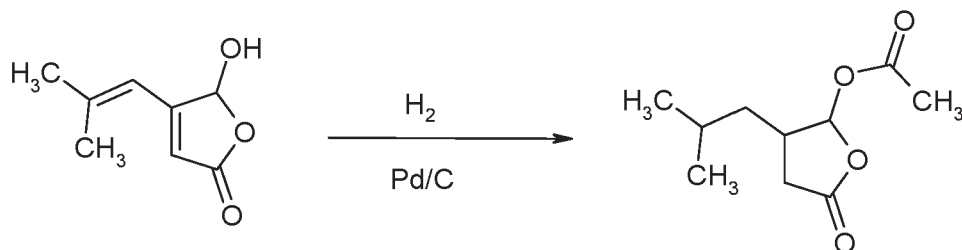


A suspension of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one (19g, 0.123mol) in ethyl acetate (100mL) was treated with solid sodium carbonate (13.1g, 0.123mol) and tetrabutylammonium hydrogensulfate (0.5g). Acetic anhydride (18.8g, 1.5eq.) was added in one portion. A mildly exothermic reaction ensued. The mixture was stirred overnight, water (100mL) added and the organic phase separated (pH of aqueous phase was 6.0). The ethyl acetate phase was water washed and concentrated to leave a solid (24.6g, 100%). This was pure by TLC (100:3 CPME: acetic acid). If this reaction is carried out in isopropyl acetate, the pure compound can be isolated by cooling the isopropyl acetate solution after the water washing in about 70% yield.

m/z: 196

Example 16

Hydrogenation of 5-acetoxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one

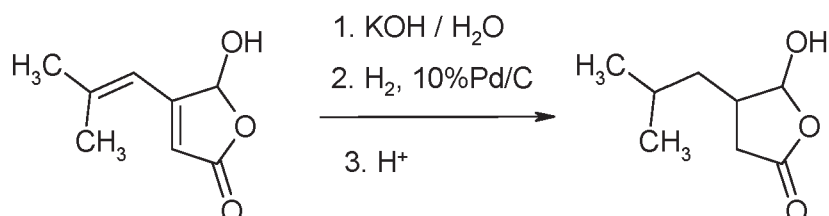


The material of example 34 was hydrogenated under similar conditions to those outlined in Example 13 to give a >90% yield of 5-acetoxy-4-(2-methylpropyl)-3,4-dihydro-2(5H)-furanone as a 1:1 mixture of diastereomers. The product is accompanied by ca. 5% of 4-(2-methylpropyl)-3,4-dihydrofuran-2(5H)-one. The product can be hydrolysed as in

Example 30 to give yields of 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone/3-formyl-5-methylhexanoic acid.

Example 17

- 5 **Preparation of 5-hydroxy-4-(2-methylpropyl)dihydrofuran-2(3H)-one (I^A) by hydrogenation of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one in alkaline solution**

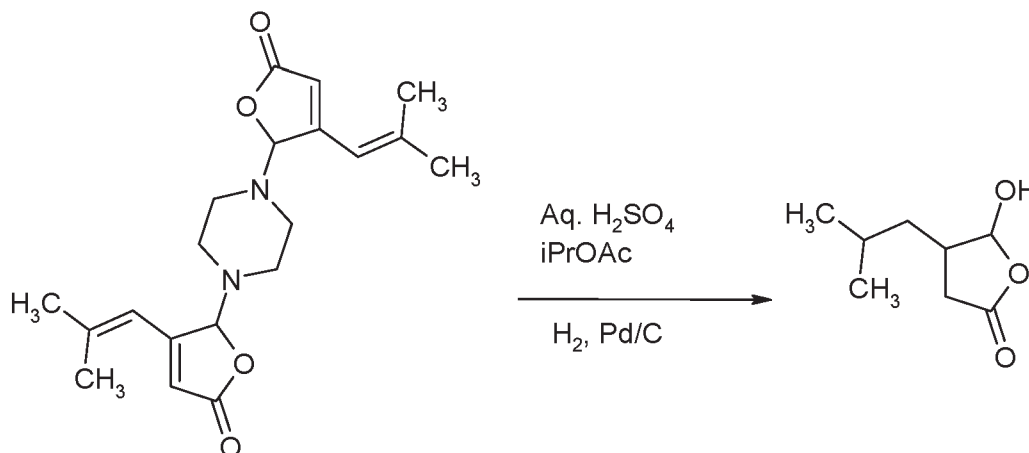


- 10 5-Hydroxy-4-(2-methylpropen-1-yl)-2-furanone (3.35g, 21.7mmol) and water (20mL) were charged to a hydrogenator. Potassium hydroxide (1.21g, 1eq) was then charged to the vessel and the contents were heated to 40°C until dissolution had occurred. 10% Pd/carbon catalyst (0.67g) was charged to the vessel and the reactor contents were then hydrogenated at 40°C and 5barg hydrogen pressure. On completion of reaction the reaction was cooled and the catalyst removed by filtration. The pH was adjusted to pH 2
- 15 by addition of 36% hydrochloric acid and the aqueous layer was washed with toluene to extract the desired product. The combined toluene extracts were concentrated to give the title compound (3.17g, 92%).

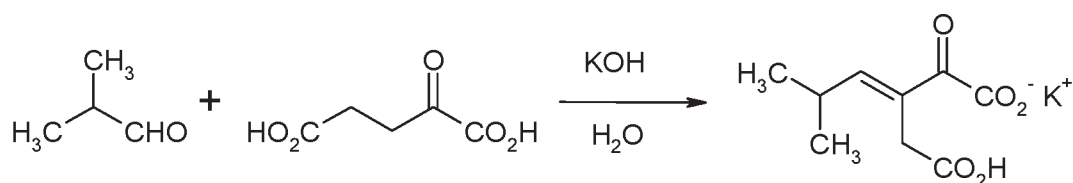
Example 18

- 20 **Hydrogenation of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one in neutral solution.**

- 5-Hydroxy-4-(2-methylpropen-1-yl)-2-furanone was hydrogenated in 6mL of water per gram starting material with 10% w/w of Pd/C catalyst. (22h, 40°C, 10 bar). On filtration, an oily phase separated from the water. This was a 1:1 mixture of 5-hydroxy-4-(2-methylpropyl)dihydrofuran-2(3H)-one (I^A) and 4-(2-methylpropyl)-dihydrofuran-2-one.
- 25 Compound (I^A) can easily be separated pure by an acid base extraction. This reaction was repeated in organic solvents with 2-propanol giving relatively pure (I^A).

Example 19**Preparation of 5-hydroxy-4-(2-methylpropyl)dihydrofuran-2(3H)-one (I^A) from 5,5'-(piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) in one pot**

- 5 5,5'-(Piperazine-1,4-diyl)bis(4-(2-methylprop-1-en-1-yl)furan-2(5H)-one) (50.0g, 0.14mol) was charged to water (300mL) containing sulphuric acid (16.0g, 0.163mol). Isopropyl acetate (200mL) was added. 50% water wet 5% palladium on carbon (3.0g) was added and the mixture was hydrogenated (5bar hydrogen) at ambient temperature for 15h. The reaction mixture was filtered from catalyst, washed with isopropyl acetate
- 10 (300mL) – this was also used to wash out the vessel. The organic phase was separated, washed with water (100mL) and concentrated on a rotary evaporator to afford 38.8g of clear oil. This was a mixture of desired compound (I^A) and the over-reduced lactone (4-(2-methylpropyl)-dihydrofuran-2-one). It was readily purified by extraction with aqueous potassium carbonate solution and wash with toluene.
- 15 Acidification of the potassium carbonate extract with formic acid gave the desired product (I^A) (25.6 g, 58%).
- ¹H NMR (D₂O with added K₂CO₃): δ 9.5 (1H), 2.8 (m, 2H), 2.40 (m, 2H), 1.55 (m, 2H), 1.30 (m, 2H), 0.95 (m, 6H).

Example 20**Preparation of 3-isobutylidene-2-oxopentanedioic acid mono-potassium salt.**

A 2L round bottom was charged 300g α-ketoglutaric acid and 450mL of ice water. With stirring and cooling 314g of potassium hydroxide in 450mL water was charged keeping

the pot temperature less than 25°C. Reactor was placed under nitrogen and isobutyraldehyde was added at 0.2mL/min over 50hrs. The resulting two phase yellow solution was separated and washed with 100mL MTBE. The lower aqueous phase pH was adjusted to 3.2-3.4 and was concentrated to 1/3 volume at 65°C under vacuum.

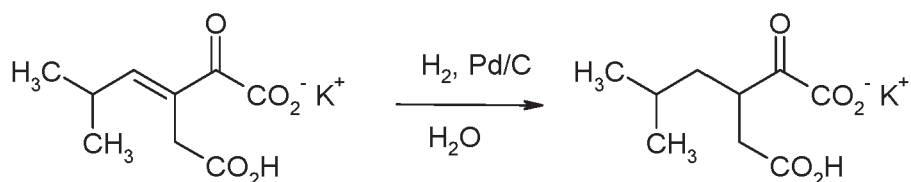
- 5 After cooling to <5°C the resulting solids were collected and washed with several small volumes of water to give after drying 230g of a white solid containing 60% 3-isobutyridene-2-oxopentanedioic acid mono-potassium salt and remainder KCl.

¹H NMR (D₂O at pH 8-10, 400MHz) δ 6.4 (d, 1H), 3.3 (s, 2H), 2.7 (m, 1H), 0.96 (d, 6H).

- 10 ¹³C NMR (D₂O at pH 8-10, 400MHz) δ 23 (2CH₃), 31 (CH), 35 (CH₂), 133 (C), 164 (CH), 170 (C), 176 (C), 182 (C).

Example 21

Preparation of 3-(2-methylpropyl)-2-oxopentanedioic acid mono-potassium salt



- 15 An aqueous solution of 3-isobutyridene-2-oxopentanedioic acid mono-potassium salt as generated in example 20 was adjusted to pH 6-8 and 5% Pd/C was added and the solution was hydrogenated at 10bar H₂ 25°C for 10hours. The catalyst was removed by filtration and the pH of the solution adjusted to pH 3.8. The mixture was cooled to <5°C and filtered and washed with a small amount of cold water. The resulting white crystals
- 20 were dried at 40°C under vacuum to give the title compound, 286g, 58% yield over 2 steps.

¹H NMR (D₂O at pH 8-10, 400MHz) δ 3.5 (m, 1H), 2.5 (dd, 1H), 2.3 (dd, 1H), 1.6-1.5 (m, 2H) 1.3-1.2 (m, 1H) 0.9 (d, 6H). ¹³C NMR (D₂O at pH 8-10, 400MHz) δ 21.7 (CH₃),

- 25 22.2 (CH₃), 25.5 (CH), 38.4 (CH₂), 39.3 (CH₂), 43.4 (CH), 167.8 (C), 170.6 (C), 180.7 (C).

Example 22

Expression of Decarboxylase Enzymes in *E. coli*

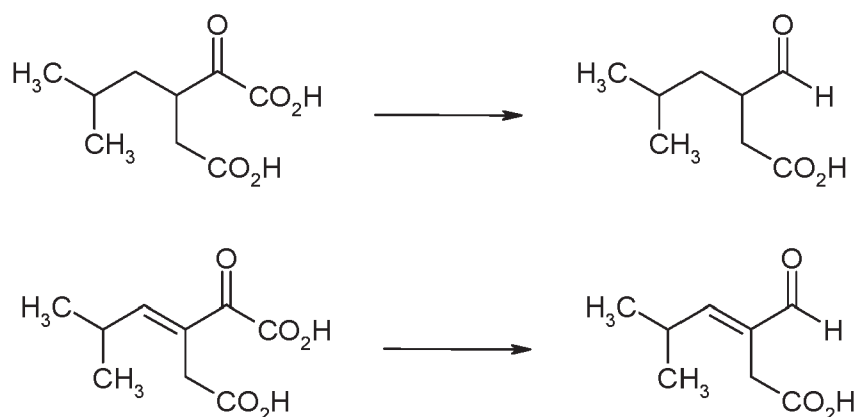
- 30 PubMed was used to search the literature for decarboxylase enzymes with a broad spectrum of activity. The KEGG (Kyoto Encyclopedia of Genes and Genomes) program was used to search for microbial decarboxylases with activity on compounds somewhat similar in structure to 3-(2-methylpropyl)-2-oxopentanedioic acid or 3-isobutyridene-2-

oxopentanedioic acid. Seven classes of decarboxylases were chosen for investigation based on reports of activity on compounds with some structural similarity. The decarboxylase classes were glutamate decarboxylase, diaminopimelate decarboxylase, indolepyruvate decarboxylase, branched-chain α -keto acid decarboxylase, aromatic-L-amino-acid decarboxylase, lysine decarboxylases and benzoylformate decarboxylase. Forty one sequences of genes for proven, or putative decarboxylase enzymes were selected. The genes were codon optimized for expression in *E. coli*, synthesized by GeneArt (Germany), DNA2.0 (Menlo Park, CA USA), or Blue Heron Biotechnology (Bothell, WA USA), cloned into expression vector pSTRC52 (Pfizer Inc., USA), and put into the expression strain *E. coli* BDG62 (Pfizer Inc., USA) (Table 1 below). Two genes were amplified from genomic DNA from the appropriate organisms by PCR and cloned into the same expression vector and *E. coli* strain. Four decarboxylase enzymes were purchased from Sigma and tested for activity on 3-(2-methylpropyl)-2-oxopentanedioic acid.

Each *E. coli* strain containing the cloned decarboxylase gene was grown overnight in LB broth with the appropriate antibiotic. A small amount (50 μ L – 100 μ L) of the overnight seed culture was used to inoculate 4.0mL of Terrific Broth medium with appropriate antibiotics in 20mm diameter culture tubes. The cultures were grown in a shaking incubator at 32°C and 300rpm. After 5h of growth IPTG was added to 0.4mM final concentration to induce the enzyme expression. The cultures were returned to the incubator and grown for an additional 19h.

Example 23

Decarboxylation of 3-(2-methylpropyl)-2-oxopentanedioic acid potassium salt and 3-isobutylidene-2-oxopentanedioic acid potassium salt with Decarboxylase Enzymes



Recombinant decarboxylase enzymes were tested for decarboxylation of 3-(2-methylpropyl)-2-oxopentanedioic acid (**XV**) and 3-isobutylidene-2-oxopentanedioic acid (**XII**) using *E. coli* cells prepared as described in Example 22. Reactions (1mL) were carried out at 37°C in potassium phosphate buffer (100mM, pH 6.4) with decarboxylase (60mg wet cells), 50mM 3-(2-methylpropyl)-2-oxopentanedioic acid or 3-isobutylidene-2-oxopentanedioic acid, ThDP (0.1mM), and MgSO₄ (2.5mM). Decarboxylation of 3-(2-methylpropyl)-2-oxopentanedioic acid and 3-isobutylidene-2-oxopentanedioic acid was determined by a UPLC assay. An aliquot (0.1mL) of the reaction was treated with 0.5mL of potassium phosphate buffer (100mM, adjusted to pH 2.2 with phosphoric acid) and 0.3mL of a solution of 2,4-dinitrophenylhydrazine (20mM in 1M HCl:acetonitrile, 3:1, v/v) for 30min at 50°C. The derivatized samples were diluted with 0.5mL of acetonitrile, filtered and analyzed by UPLC on a Agilent Eclipse Plus C18 column (100mm x 3.0mm, 1.8µm) eluted with 0.1% trifluoroacetic acid in water:acetonitrile (55:45, v/v) at 1.1mL/min. The column was maintained at 40°C and the effluent was monitored at 360nm and ES⁺ mass spectroscopy. Positive results were indicated by the presence of a peak for 3-formyl-5-methylhexanoic acid from 3-(2-methylpropyl)-2-oxopentanedioic acid or 3-formyl-5-methylhex-3-enoic acid from 3-isobutylidene-2-oxopentanedioic acid. Results of the analyses are shown in Table 1.

Table 1

Enzyme Name	Source Organism	Reaction on (XV)	Reaction on (XII)
Glutamate decarboxylases			
Aa gad	<i>Arthrobacter aurescens</i>	— ^a	NT ^b
Rs gad	<i>Rhodococcus</i> sp. RHA1	—	NT
Sc gad	<i>Streptomyces coelicolor</i>	—	NT
Dz gad	<i>Dickeya zeae</i>	—	NT
Pa gad	<i>Pyrobaculum arsenaticum</i>	—	NT
Mm gad	<i>Mycobacterium marinum</i>	—	NT
UT gad	UTI89 metagenomic DNA	—	NT
Rm gad	<i>Ralstonia metallidurans</i>	—	NT
Re gad	<i>Ralstonia eutropha</i>	—	NT
Xn gad	<i>Xenorhabdus nematophila</i>	—	NT
Pd gad	<i>Photobacterium damsela</i>	—	NT

Enzyme Name	Source Organism	Reaction on (XV)	Reaction on (XII)
Branched-chain α-Keto Acid decarboxylase			
Pa bkd	<i>Photorhabdus asymbiotica</i>	—	—
Sv bkdA	<i>Streptomyces virginiae</i>	—	—
LI kdcA	<i>Lactococcus lactis</i>	+	+
Indolepyruvate decarboxylases			
Ss ipd	<i>Staphylococcus saprophyticus</i>	—	+
Dd ipd	<i>Desulfovibrio desulfuricans</i>	+	+
Bm ipd	<i>Bacillus megaterium</i>	—	—
Dv ipd	<i>Desulfovibrio vulgaris</i>	—	—
Aa ipd	<i>Aromatoleum aromaticum</i>	—	—
Ab ipd	<i>Azospirillum brasilense</i>	—	—
Bs ipd	<i>Bradyrhizobium</i> sp. BTAi1	—	—
NC ipd	NC10 bacterium	—	—
Rr ipd	<i>Rhodospirillum rubrum</i>	—	—
Aromatic-L-amino-acid decarboxylases			
Ab dcd	<i>Acinetobacter baumannii</i>	—	—
Bp dcd	<i>Bacillus pumilus</i>	—	—
Cg dcd	<i>Chryseobacterium gleum</i>	—	—
As dcd	<i>Anaeromyxobacter</i> sp. Fw109-5	—	—
Bs dcd	<i>Burkholderia</i> sp. 383	+	—
Cs dcd	<i>Cyanothece</i> sp. PCC 8801	—	—
Lysine decarboxylases			
Cr lysA	<i>Candidatus Carsonella ruddii</i>	—	—
Dt lysA	<i>Dictyoglomus thermophilum</i>	—	—
Sa lysA	<i>Sphingopyxis alaskensis</i>	—	—
Benzoylformate decarboxylases			
Rp bfd	<i>Rhodopseudomonas palustris</i>	+	+
Sc bfd	<i>Streptomyces coelicolor</i>	+	+
Rc bfd	<i>Ricinus communis</i>	—	—
Pp bfd	<i>Pseudomonas putida</i>	+	+
Ms bfd	<i>Mycobacterium smegmatis</i>	—	—
As bfd	<i>Arthrobacter</i> sp. FB24	+	+

Enzyme Name	Source Organism	Reaction on (XV)	Reaction on (XII)
Pb bfd	<i>Pseudomonas brassicacearum</i>	+	+
Ct bfd	<i>Comamonas testosterone</i>	+	+
Dd bfd	<i>Desulfovibrio desulfuricans</i>	+	+
Rj bfd	<i>Rhodococcus jostii</i>	+	+
Pf bfd	<i>Pseudomonas fluorescens</i>	+	+
Purified Enzymes (purchased from Sigma-Aldrich)			
L-Histidine Decarboxylase	<i>Lactobacillus</i> 30a	–	NT
L-Lysine Decarboxylase	<i>Bacterium cadaveris</i>	–	NT
L-Tyrosine Decarboxylase	<i>Streptococcus faecalis</i>	–	NT
Pyruvate Decarboxylase	<i>Saccharomyces cerevisiae</i>	–	NT

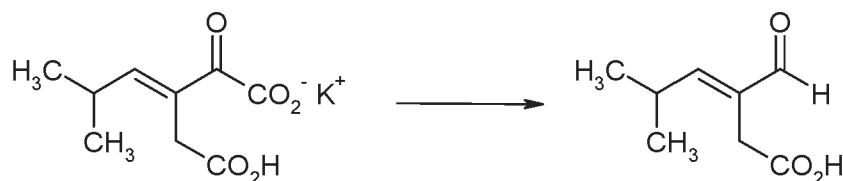
^a –: No detection of appropriate product

^b NT: Not tested

^c +: Detection of appropriate product

5 **Example 24**

Preparation of 3-Formyl-5-methylhex-3-enoic acid



In a 250mL round bottom flask 80g of cell concentrate comprising *E. Coli* expressing *Pseudomonas putida* benzoylformate decarboxylase (see example 23) was charged.

- 10 To this was added a pH 6.2 adjusted solution of 3-isobutylidene-2-oxopentanedioic acid mono-potassium salt (10g, see example 20) in 50mL water, with 0.5g of magnesium sulfate and 0.5g of thiamine pyrophosphate. The resulting pH 6.2 slurry was heated to 50°C and stirred for 145hrs keeping the pH adjusted between 6.2 and 7.2 with concentrated HCl. The reaction mixture was cooled to RT and centrifuged. The
- 15 aqueous decants were washed with 50mL MTBE with minimum agitation. The pH of

the aqueous phase was adjusted to 4 and filtered through celite. The filtrate was extracted with minimum agitation three times with 50mL MTBE. The MTBE was dried over anhydrous sodium sulfate and concentrated to a thick red oil. This oil was extracted with several portions of hot hexanes; the combined hexanes were cooled to
5 <0° and the resulting crystals were isolated and dried in air to give 3-formyl-5-methylhex-3-enoic acid as a white solid (0.6g).

¹H NMR (D₂O at pH 8-10, 400MHz) δ 9.2 (s, 1H), 6.6 (d, 1H), 3.1 (s, 2H), 2.7 (m, 1H), 1 (d, 6H)

10 **Example 25**

Expression of Enoate Reductase Homologues in *E. coli*

The DNA sequence corresponding to the gene for *Lycopersicon esculentum* (tomato) 12-oxophytodienoate reductase 1 (OPR1) was retrieved from the Genbank database (accession number AC Q9XG54) and was synthesized by GeneArt (Germany). The
15 sequence was codon optimized for expression in *E. coli*, and was subcloned into an *E. coli* expression plasmid pSTRC18 (Pfizer Inc., USA). The protein sequence is shown below. The OPR1 expression construct was transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were incubated in LB + streptomycin media. The LB culture was used to
20 inoculate expression cultures (LB, M9Y, or TB), which were incubated at 37°C (210rpm) After the culture reached a suitable biomass concentration (OD 1 at A600), IPTG was added (1mM) and cultures were incubated for another 20 h (30°C, 210 rpm). The cells were harvested by centrifugation (4000 x g, 30 min, 4°C) and stored at -20 °C.

The BLASTP program was used to search the NCBI non-redundant protein sequences
25 database for gene sequences related to 12-Oxophytodienoate reductase (OPR1) from *Lycopersicon esculentum*. Thirty eight sequences for related genes were selected, codon optimized for expression in *E.coli*, and subcloned into the pET28b(+) *E. coli* expression plasmid (Novagen, EMD Chemicals, Gibbstown, NJ, USA). The OPR1 related expression constructs were transformed into BL21(DE3) *E. coli* (Stratagene,
30 Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB Medium (Novagen, EMD Chemicals, Gibbstown, NJ, USA). Cultures were incubated for 20h at 30°C, and the cells were harvested by centrifugation (4000 x g, 30min, 4°C) and stored at -20 °C.

Lycopersicon esculentum (tomato) 12-Oxophytodienoate Reductase 1 protein sequence:

```

MENKVVEEKQ  VDKIPLMSPC  KMGKFELCHR  VVLAPLTRQR  SYGYIPQPHA
ILHYSQRSTN  GLLIGEATV   ISETGIGYKD  VPGIWTKEQV  EAWKPIVDAV
5  HAKGGIFFCQ  IWHVGRVSNK  DFQPNGEDPI  SCTDRGLTPQ  IRSNGIDIAH
FTRPRRLTTD  EIPQIVNEFR  VAARNAIEAG  FDGVEIHGAH  GYLIDQFMKD
QVNDRS DKYG  GSLENRCRFA  LEIVEAVANE  IGSDRVGIRI  SPFAHYNEAG
DTNPTALGLY MVESLNKYDL AYCHVVEPRM KTAWEKIECT ESLVPMRKAY
KGTFIVAGGY  DREDGNRALI  EDRADLVAYG  RLFISNPDL  KRFELNAPLN
10 KYNRDTFYTS DPIVGYTDYP FLETMT (SEQ ID NO. 8)

```

Lycopersicon esculentum (tomato) 12-Oxophytodienoate Reductase 1 codon optimized sequence:

```

ATGGAAAACAAAGTTGTGGAAGAAAAACAGGTTGATAAAATCCCGCTGATGAGCCC
15 GTGTAAATGGGTAAATTCGAGCTGTGTCATCGCGTTGTACTGGCACCGCTGACTC
GTCAGCGTTCTTATGGTTACATTCCGCAGCCGCACGCAATCCTGCATTACTCTCAG
CGCAGCACCAACGGTGGCCTGCTGATCGGTGAAGCAACCGTGATCAGCGAAACT
GGCATCGGTTACAAAGATGTGCCGGGTATCTGGACGAAAGAGCAGGTTGAGGCCT
GGAAACCGATCGTCGACGCGGTGCATGCCAAAGGTGGTATTTTCTTTTGTGAGATC
20 TGGCACGTTGGTCGTGTATCCAACAAAGATTTTCAGCCGAACGGCGAAGATCCGA
TTTCCTGTACTGACCGCGGCCTGACCCCGCAGATCCGTTCCAACGGCATTGACAT
TGCCCACTTCACCCGTCCACGTGCGCTGACTACTGACGAGATTCCGCAGATCGTG
AACGAGTTCCGCGTTGCAGCGCGTAATGCTATTGAAGCGGGTTTCGATGGCGTCG
AGATTCATGGTGCCACGGTTACCTGATCGACCAATTCATGAAAGACCAAGTTAAC
25 GACCGCAGCGATAAGTATGGCGGTTCTCTGGAGAACCGTTGTCGCTTCGCGCTGG
AAATCGTTGAAGCAGTAGCCAACGAGATTGGCTCCGACCGTGTTGGTATCCGTATC
TCTCCATTCGCACACTACAACGAAGCGGGCGACACTAACCCGACCGCACTGGGCC
TGTATATGGTGGAGAGCCTGAATAAATACGACCTGGCGTATTGTCACGTGGTCTGA
GCCGCGCATGAAAACCGCCTGGGAAAAGATTGAGTGACCCGAAAGCCTGGTGCC
30 GATGCGTAAAGCCTACAAAGGCACCTTCATCGTAGCTGGTGGCTACGACCGTGAA
GACGGTAACCGCGCTCTGATCGAAGACCGTGCCGACCTGGTTGCGTACGGTCGT
CTGTTTCATCAGCAACCCAGACCTGCCGAAGCGTTTTGAACTGAACGCTCCGCTGA
ACAAATACAACCGTGACACTTTCTACACTTCCGACCCGATCGTTGGTTACACCGAT
TACCCGTTTCTGGAAACTATGACTTAATAA (SEQ ID NO. 9)

```

Example 26**Reduction of (E)-3-formyl-5-methylhex-3-enoic acid with Recombinant Reductases**

- 5 Recombinant enoate reductases were tested for reduction of (E)-3-formyl-5-methylhex-3-enoic acid using *E. coli* cells prepared as described in Example 25. Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH 7.0) with *E. coli* cells (100mg wet cells/mL), NADPH (10mM), NADH (10mM) and (E)-3-formyl-5-methylhex-2-enoic acid (10mM). After 16 h, acetonitrile (0.5ml) was added to each
- 10 reaction and the resulting mixtures were centrifuged (2000rpm x 5min). Aliquots (0.1mL) of the resulting supernatants were treated with 0.1mL of potassium phosphate buffer (100mM, adjusted to pH 2.2 with phosphoric acid) and 0.225mL of a solution of 2,4-dinitrophenylhydrazine (20mM in 1M HCl:acetonitrile, 3:1, v/v) for 30min at 50°C. The derivatized samples were diluted with 0.225mL of acetonitrile and analyzed by
- 15 HPLC on a Phenomenex Lux 5 μ Amylose-2 column (250mm x 4.6mm id) eluted with 0.1% trifluoroacetic acid in water:acetonitrile (65:35, v/v) at 2mL/min. The column was maintained at 50°C and the effluent was monitored at 360nm. Results of HPLC analyses are shown in Table 2.

20

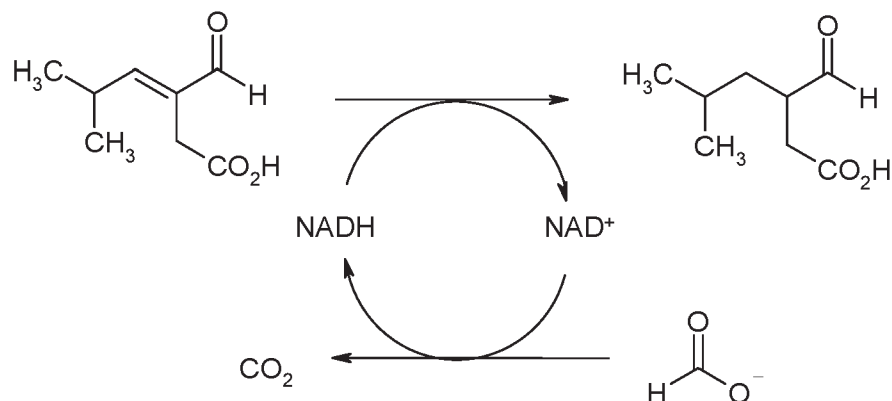
Table 2

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
1	Old yellow enzyme 1 / <i>Saccharomyces carlsbergensis</i>	Q02899	6.5
2	Old yellow enzyme 2 / <i>Saccharomyces cerevisiae</i>	Q03558	9.2
3	Old yellow enzyme 3 / <i>Saccharomyces cerevisiae</i>	P41816	8.6
4	NADH:flavin oxidoreductase / <i>Zymomonas mobilis</i>	Q5NLA1	85.0

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
5	fumarate reductase / <i>Shewanella frigidimarina</i>	Q07WU7	3.4
6	Pentaerythritol tetranitrate reductase / <i>Enterobacter cloacae</i>	Q6JL81	85.6
7	Unnamed enzyme / <i>Arabidopsis thaliana</i>	BAH57049	3.7
8	Allyl alcohol dehydrogenase / <i>Nicotiana tabacum</i>	BAA89423	5.0
9	Artemisinic aldehyde delta-11(13) reductase / <i>Artemisia annua</i>	1WLY_A	37.1
10	2-haloacrylate reductase / <i>Burkholderia</i> sp. WS	NP_390263	4.2
11	NADPH dehydrogenase / <i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	YP_390263	82.8
12	NADH:flavin oxidoreductase/NADH oxidase / <i>Thermoanaerobacter pseudoethanolicus</i>	YP_001664021	54.9
13	Unnamed enzyme / <i>Clostridium tyrobutyricum</i>	CAA71086	6.0
14	Unnamed enzyme / <i>Moorella thermoautotrophica</i>	Q2RGT7	4.8
15	oxophytodienoate reductase (OPR1) / <i>Lycopersicon esculentum</i>	Q9XG54	31.4
16	Oxophytodienoate reductase (OPR3) / <i>Lycopersicon esculentum</i>	Q9FEW9	15.7
17	N-ethylmaleimide reductase / <i>Shigella sonnei</i>	Q3Z206	100.0

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
18	12-oxo-phytodienoic acid reductase / <i>Zea mays</i>	Q49HE0	100.0
19	12-oxo-phytodienoic acid reductase / <i>Zea mays</i>	Q49HE4	100.0
20	Unnamed enzyme / <i>Vitis vinifera</i>	A5BF80	7.5
21	Unnamed enzyme / <i>Populus trichocarpa</i>	B9MWG6	100.0
22	12-oxophytodienoate reductase / <i>Arabidopsis thaliana</i>	Q8GYB8	100.0
23	12-oxophytodienoate reductase / Castor bean	B9SK95	5.4
24	NADH:flavin oxidoreductase/NADH oxidase / <i>Klebsiella variicola</i>	D0YIM0	100.0
25	Unnamed enzyme / <i>Citrobacter koseri</i>	A8AH31	100.0
26	N-ethylmaleimide reductase / <i>Citrobacter sp.</i>	C1M473	100.0
27	N-ethylmaleimide reductase / <i>Citrobacter rodentium</i>	D2THI8	100.0
28	N-ethylmaleimide reductase / <i>Salmonella paratyphi</i>	Q5PH09	100.0
29	N-ethylmaleimide reductase / <i>Cronobacter turicensis</i>	C9Y3L1	100.0
30	Unnamed enzyme / <i>Providencia stuartii</i>	B2Q290	100.0
31	NADPH dehydrogenase / <i>Yarrowia lipolytica</i>	Q6CI57	9.1

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
32	N-ethylmaleimide reductase / <i>Pseudomonas putida</i>	Q88I29	17.4
33	12-oxophytodienoate reductase / <i>Pichia stipitis</i>	150864790	7.8
34	NAPDH dehydrogenase / <i>Pichia stipitis</i>	126131638	12.6
35	Unnamed enzyme / <i>Pichia guilliermondii</i>	146393506	21.6
36	unnamed enzyme / <i>Candida glabrata</i>	50293551	36.5
37	Unnamed enzyme / <i>Debaryomyces hansenii</i>	50405397	9.1
38	Unnamed enzyme/ <i>Geobacillus kaustophilus</i> HTA426	Q5KXG9	100.0
39	Oxophytodienoate reductase (OPR2) / <i>Lycopersicon esculentum</i>	Q9XG54	14.0
¹ %Conversion of (E)-3-formyl-5-methylhex-2-enoic acid to 3-formyl-5-methylhexanoic acid			

Example 27**Reduction of (*E*)-3-formyl-5-methylhex-3-enoic acid with Recombinant Reductases and Formate Dehydrogenase**

- 5 Recombinant enoate reductases were evaluated for reduction of (*E*)-3-formyl-5-methylhex-3-enoic acid with NAD⁺ and formate dehydrogenase. Enoate reductases were expressed in *E. coli* cells as described in Example 25. Formate dehydrogenase was expressed in *E. coli* cells as follows: The pET26b formate dehydrogenase expression construct was transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB Medium + kanamycin (Novagen, EMD Chemicals, Gibbstown, NJ, USA). Cultures were incubated for 20 h at 30°C, and the cells were harvested by centrifugation (4000 x *g*, 30 min, 4°C) and stored at -20 °C.
- 10 A variant (D74M) of oxophytodienoate reductase 3 (Accession number Q9FEW9) was expressed in *E. coli* cells as follows: QuikChange Site-directed Mutagenesis kit from Stratagene (La Jolla, CA, USA) was used to create oxophytodienoate reductase 3 variant D74M as directed. Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). The pSTRC18 oxophytodienoate reductase 3 (OPR3) expression construct was transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in expansion broth + streptomycin (Zymo Research, Irvine, CA, USA). The LB cultures were used to inoculate expression cultures grown in Overexpression broth + streptomycin (Zymo Research, Irvine, CA, USA). Cultures were incubated for 20h at
- 25 23°C, and the cells were harvested by centrifugation (4000 x *g*, 30min, 4°C) and stored at -20°C.

Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH 7.0) with enoate reductases (40mg wet cells/mL), formate dehydrogenase (80mg wet

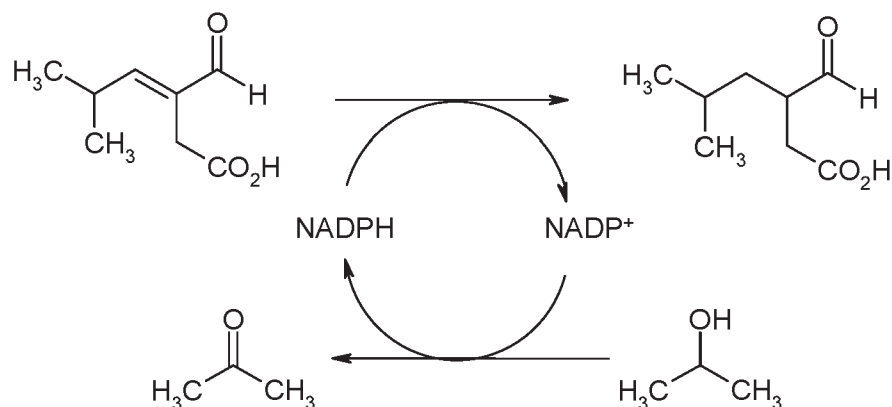
cells/mL), NAD⁺ (0.02mM), ammonium formate (30mM) and (*E*)-3-formyl-5-methylhex-3-enoic acid (20mM). After 24 h, reaction mixtures were acidified with 0.025mL of 4N HCl and extracted with 1mL of ethyl acetate. Aliquots (0.5mL) of the ethyl acetate extracts (0.5mL) were dried over anhydrous sodium sulfate and treated with methanol (0.02mL) and (trimethylsilyl)diazomethane (0.01mL of a 2M solution in diethyl ether) to derivatize carboxylic acid moieties to their corresponding methyl esters. The derivatized samples were analyzed by GC on a Chiraldex™ G-TA column (30M x 0.25mm column, column temperature: 135°C isothermal, injector temperature: 200°C, carrier gas: helium, flow rate approximately 1mL/min) to give the results shown in Table 3.

10

Table 3

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
1	NADH:flavin oxidoreductase / <i>Zymomonas mobilis</i>	Q5NLA1	23.8
2	Pentaerythritol tetranitrate reductase / <i>Enterobacter cloacae</i>	Q6JL81	19.6
3	NADPH dehydrogenase / <i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	YP_390263	17.0
4	NADH:flavin oxidoreductase/NADH oxidase / <i>Thermoanaerobacter pseudoethanolicus</i>	YP_001664021	15.9
5	oxophytodienoate reductase (OPR 3) variant D74M / <i>Lycopersicon esculentum</i>		43.5
6	N-ethylmaleimide reductase / <i>Shigella sonnei</i>	Q3Z206	24.0
7	Unnamed enzyme / <i>Populus trichocarpa</i>	B9MWG6	31.8
8	N-ethylmaleimide reductase / <i>Citrobacter</i> sp. 30_2	C1M473	22.8
9	N-ethylmaleimide reductase /	C9Y3L1	26.3

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
	<i>Cronobacter turicensis</i> DSM 18703		
10	Unnamed enzyme / <i>Geobacillus kaustophilus</i> HTA426	Q5KXG9	21.7
11	12-oxophytodienoate reductase (OPR1) / <i>Lycopersicon esculentum</i>	Q9XG54	39.8
¹ %Conversion of (<i>E</i>)-3-formyl-5-methylhex-2-enoic acid to 3-formyl-5-methylhexanoic acid (all reactions gave approximately 1:1 mixtures of (<i>S</i>)- and (<i>R</i>)-enantiomers)			

Example 28**Reduction of (*E*)-3-formyl-5-methylhex-3-enoic acid with Recombinant****5 Reductases**

Recombinant enoate reductases were evaluated for reduction of (*E*)-3-formyl-5-methylhex-3-enoic acid with NADP⁺ and *Lactobacillus brevis* alcohol dehydrogenase (X-zyme). Enoate reductases were expressed in *E. coli* cells as described in Example 25.

- 10 A variant (D74M) of oxophytodienoate reductase 3 (OPR3) was prepared as described in Example 27. Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH 7.0) with enoate reductases (40mg wet cells/mL), *Lactobacillus brevis* alcohol dehydrogenase (32U/mL), NADP⁺ (0.02mM), 2-propanol (3 vol%) and (*E*)-3-formyl-5-methylhex-2-enoic acid (20mM). After 24 h, reaction mixtures were
- 15 acidified with 0.025mL of 4N HCl and extracted with 1mL of ethyl acetate. Aliquots (0.5mL) of the ethyl acetate extracts (0.5mL) were dried over anhydrous sodium sulfate and treated with methanol (0.02mL) and (trimethylsilyl)diazomethane (0.01mL of a 2M

solution in diethyl ether) to derivatize carboxylic acid moieties to their corresponding methyl esters. The derivatized samples were analyzed by GC on a Chiraldex™ G-TA column (30M x 0.25mm column, column temperature: 135°C isothermal, injector temperature: 200°C, carrier gas: helium, flow rate approximately 1mL/min) to give the

5 results shown in Table 4.

Table 4

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
1	NADH:flavin oxidoreductase / <i>Zymomonas mobilis</i>	Q5NLA1	73.0
2	Pentaerythritol tetranitrate reductase / <i>Enterobacter cloacae</i>	Q6JL81	100.0
3	NADPH dehydrogenase / <i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	YP_390263	100.0
4	NADH:flavin oxidoreductase/NADH oxidase / <i>Thermoanaerobacter pseudoethanolicus</i>	YP_001664021	100.0
5	oxophytodienoate reductase (OPR 3) variant D74M / <i>Lycopersicon esculentum</i>		96.8
6	N-ethylmaleimide reductase / <i>Shigella sonnei</i>	Q3Z206	100.0
7	Unnamed enzyme / <i>Populus trichocarpa</i>	B9MWG6	100.0
8	N-ethylmaleimide reductase / <i>Citrobacter sp.</i> 30_2	C1M473	100.0
9	N-ethylmaleimide reductase / <i>Cronobacter turicensis</i> DSM 18703	C9Y3L1	99.5
10	Unnamed enzyme / <i>Geobacillus kaustophilus</i> HTA426	Q5KXG9	100.0

Entry	Enzyme Name /Source	Accession Number	% Conversion ¹
11	12-oxophytodienoate reductase (OPR1) / <i>Lycopersicon esculentum</i>	Q9XG54	34.3
¹ %Conversion of (<i>E</i>)-3-formyl-5-methylhex-2-enoic acid to 3-formyl-5-methylhexanoic acid (all reactions gave approximately 1:1 mixtures of (<i>S</i>)- and (<i>R</i>)-enantiomers)			

Example 29

Preparation of 3-formyl-5-methylhexanoic acid

A reaction vessel was charged with 7.5mL potassium phosphate buffer (0.1M, pH 7.0), 8.4mg NADP⁺, 0.2mL *Lactobacillus brevis* alcohol dehydrogenase (32U/mL, X-zyme), 0.3mL 2-propanol, pentaerythritol tetranitrate reductase (2mL of a 200mg/mL suspension of *E. coli* cells in potassium phosphate buffer), and 156mg of (*E*)-3-formyl-5-methylhex-3-enoic acid, and agitated at 40°C. After 6.75h, the reaction mixture was centrifuged and the supernatant was adjusted to pH 2 with 4N HCl and extracted with ethyl acetate (2 x 10mL). The ethyl acetate extract was dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure to give 141mg of colorless oil (89% yield).

¹H NMR (D₂O at pH 8 – 10) δ 2.53 – 2.43 (m, 2 H), 2.34 – 2.28 (m, 1H), 1.53 – 1.42 (m, 1H), 1.41 – 1.34 (m, 1H), 1.20 – 1.12 (m, 1H), 0.76 (d, 3H), 0.74 (d, 3H).

Example 30

Biotransformation of 3-formyl-5-methylhexanoic acid to pregabalin with recombinant ω-transaminases.



Transamination of 3-formyl-5-methylhexanoic acid to form Pregabalin was evaluated with various recombinant transaminases. Recombinant ω-transaminases from *Vibrio fluvialis*, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans* were expressed in *E. coli* as follows: The pET28b ω-transaminase expression constructs were transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as

directed and overnight cultures were grown in LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB + kanamycin Medium (Novagen, EMD Chemicals, Gibbstown, NJ, USA). Cultures were incubated for 20 h at 30°C, and the cells were harvested by centrifugation (4000 x g, 30 min, 4°C) and stored at -20°C.

Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH 7.0), pyridoxal phosphate (2mM), isopropylamine (150mM), 3-formyl-5-methylhexanoic acid (50mM) and ω-transaminase (40mg wet cells/mL) from *Vibrio fluvialis*, *Rhodobacter sphaeroides*, or *Paracoccus denitrificans*. After 24h, reaction samples (0.1mL) were diluted with 0.4mL acetonitrile:water (1:1, v/v). Aliquots (0.05mL) of the diluted reaction samples were treated with saturated aqueous sodium bicarbonate (0.01mL) and Marfey's reagent (*N*-α-(2,4-dinitro-5-fluorophenyl)alaninamide, 0.2mL of 5g/L solution in acetonitrile) at 40°C. After 1h, the derivatization reactions were quenched with 0.01mL 1N aqueous hydrochloric acid and diluted with 0.23mL of acetonitrile. The derivatized reaction samples were analyzed by UPLC (column: BEH C18, 50mm x 2.1mm id, gradient elution: 70% A:30% B to 55% A:45% B in 5 min (A = 1% triethylamine (pH 3 with phosphoric acid); B = acetonitrile), flow rate: 0.8mL/min, column temperature: 30°C, detection: 210-400nm) to give the results shown in Table 5.

Table 5

Entry	Enzyme	Accession Number	%Yield Pregabalin	%ee (S)- Pregabalin
1	<i>Vibrio fluvialis</i> ω-transaminase	AEA39183	37.5	60.6
2	<i>Rhodobacter sphaeroides</i> ω-transaminase	YP_001043908	24	84.4
3	<i>Paracoccus denitrificans</i> ω-transaminase	YP_917746	44	60.6

Example 31**Biotransformation of 3-formyl-5-methylhexanoic acid to pregabalin with recombinant ω -transaminases.**

5 Recombinant variants of *Vibrio fluvialis* were tested for the reductive amination of 3-formyl-5-methylhexanoic acid to form Pregabalin. Variants of *V. fluvialis* ω -transaminase (Accession number AEA39183) were expressed in *E. coli* as follows: QuikChange Site-directed Mutagenesis kits from Stratagene (La Jolla, CA, USA) was used to create *V. fluvialis* aminotransferase variants as directed. Primers were ordered from Integrated
 10 DNA Technologies (Coralville, IA, USA). The pET28b ω -transaminase expression constructs were transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB + kanamycin Medium (Novagen, EMD Chemicals,
 15 Gibbstown, NJ, USA). Cultures were incubated for 20h at 30°C, and the cells were harvested by centrifugation (4000 x g, 30min, 4°C) and stored at -20°C.

Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH 7.0) with pyridoxal phosphate (2mM), isopropylamine (300mM), 3-formyl-5-methylhexanoic acid (100mM) and *V. fluvialis* ω -transaminase wild-type or variants
 20 (40mg wet cells/mL). After 28h, reaction samples (0.1mL) were diluted with 0.4mL acetonitrile:water (1:1, v/v). Aliquots (0.05mL) of the diluted reaction samples were treated with saturated aqueous sodium bicarbonate (0.01mL) and Marfey's reagent (*N*- α -(2,4-dinitro-5-fluorophenyl)alaninamide, 0.2mL of 5g/L solution in acetonitrile) at 40°C. After 1h, the derivatization reactions were quenched with 0.01mL 1N aqueous
 25 hydrochloric acid and diluted 0.23mL of acetonitrile. The derivatized reaction samples were analyzed by UPLC (column: BEH C18, 50mm x 2.1mm id, gradient elution: 70% A:30% B to 55% A:45% B in 5min (A = 1% triethylamine (pH 3 with phosphoric acid); B = acetonitrile), flow rate: 0.8mL/min, column temperature: 30°C, detection: 210-400nm) to give the results shown in Table 6.

Table 6

Entry	<i>V. fluvialis</i> ω -transaminase variant	% Conversion	% ee
1	W57F/K163L/R415F	6.1	81.1
2	Y165F	9.5	75.3
3	W147N	10.0	75.0
4	A228G	32.3	74.9
5	N166V	16.6	74.5
6	W57F/A228G	33.3	73.9
7	V156M	13.2	70.3
8	S159A	7.9	70.2
9	W57F/R415F	10.7	68.5
10	R415F	11.8	68.1
11	M59N	10.1	68.0
12	P301K	19.1	67.6
13	Y113F	7.9	65.2
14	F86G	32.1	64.1
15	I254V	32.1	64.0
16	H326N	15.9	63.8
17	C414I	10.1	63.0
18	W57F/K163L/V153A	46.1	41.2
19	W57F/K163L	43.5	36.1
20	D21Y	45.1	34.2
21	W57F/D21Y	46.3	34.0
22	M294V	42.4	33.2
23	W57F/M294V	41.2	32.5
24	W57F/V177I	39.9	29.0
25 ¹	Vat 565	20.0	90.5
26 ¹	Vfat665	15.0	93.1
27 ¹	Vfat701	9.0	94.4
28 ¹	Vfat707	7.5	95.7
29 ¹	Vfat747	50.0	97.5
30 ¹	Vfat825	75.0	95.7
31 ¹	Vfat 850	81.3	94.6
32 ¹	Vfat 875	80.5	95.1

Entry	<i>V. fluvialis</i> ω -transaminase variant	% Conversion	% ee
33 ¹	Vfat888 ²	95.0	95.5

Note – 1: Entry 25 thru 33 – reactions were performed using 400mM of 3-formyl-5-methylhexanoic acid, 3mM PLP, 800mM IPM at 45°C.

2: Vfat 888:

DNA SEQUENCE

ATGAATAAACACAAAGCTGGGAAGCGCGTGCTGAACTTACTCTCTGTAC
 GGCTTCACTGATATGCCATCTCTGCACCAGCGTGGTACCGTGGTTGTCACC
 CACGGCGAGGGGCCCATACATCGTGGACGTCAACGGTCGCCGTTACCTGGA
 CGCAAACCTCCGGCCTGTACAATATGGTTGCCGGCTTCGACCACAAGGGTCT
 GATCGACGCAGCAAAGGCCAGTACGAACGCTTCCCGGGTTACCATAGCTT
 CTTCCGGTCGTATGTCTGATCAAACCTGTTATGCTGAGCGAGAACTGGTAGA
 GGTGTCTCCATTCGACAGCGGTGCGGTGTTCTATACTAACTCCGGCTCCGA
 GGCTAACGATACTATGGTGAAAATGCTGTGGTTTCTGCACGCCGCAGAGGG
 CAAGCCGCAAAAACGCAAAATCCTGACTCGTAACAACGCATACCACGGTGT
 AACTGCTGTTTCCGCTTCCATGACGGGTCTGCCGTACAACCTCTGTATTCGG
 CCTGCCGCTGCCGGGTTTCGTTACCTGACCTGTCCGCACTATTGGCGTTA
 CGGCGAAGAAGGTGAAACCGAAGAGCAGTTTGTTGCTCGTCTGGCCCGCG
 AGCTGGAGGAACTATCCAACGTGAAGGCGCGGACACGATTGCGGGCTTC
 TTTGCTGAGCCGGTCATGGGCGCGGGCGGCGTAATCCCGCCGGCGAAAG
 GTTACTTCCAGGCGATCCTGCCGATTCTGCGTAAGTACGACATCCCGGTTA
 TCTCTGATGAAGTTATCTGCGGCTTTGGTCGTACCGGTAATACTTGGGGTTG
 CGTTACCTATGACTTCACCCCGGATGCGATCATCTCCAGCAAAAATCTGACC
 GCCGGTTTCTTCCGGTTGGTGCTGTGATTCTGGGTCCGGAACTGGCGAAA
 CGCCTGGAAACGGCGATCGAAGCTATCGAAGAGTTCCCGCACGGCTTTAC
 GGCCAGCGGTACCCGGTGGGTTGCGCTATCGCTCTGAAAGCAATCGATG
 TTGTGATGAATGAGGGTCTGGCAGAGAACGTGCGCCGCCTGGCACCGCGT
 TTTGAGGAGCGTCTGAAACACATTGCCGAACGTCCGAACATCGGTGAATAT
 CGTGGCATCGGTTTTATGTGGGCACTGGAGGCTGTGAAAGACAAAGCATCT
 AAAACCCCATTCGATGGTAATCTGTCTGTGAGCGAGCGTATCGCTAACACCT
 GTACCGACCTGGGCCTGATCTGTAGCCCGATGGGTGAGTCCGTTATCCTGT
 GCCCGCCGTTTCATCCTGACCGAGGCGCAAATGGATGAGATGTTTGACAAAC

TGGAGAAGGCTCTGGACAAAGTCTTTGCGGAGGTGGCGTAA (SEQ ID NO. 10)

AMINO ACID SEQUENCE

MNKPQSWEARAETYSLYGFTDMPSLHQRGTVVVTHGEGPYIVDVNGRRYLDA
NSGLYNMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVS
PFDSGRVFYTNNSGSEANDTMVKMLWFLHAAEGKPQKRKILTRNNAYHGVTA
SASMTGLPYNSVFGLPLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETI
QREGADTIAGFFAEPVMGAGGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRT
GNTWGCVTYDFTPDAIISSKNLTAGFFPVGAVILGPELAKRLETAIEAIEEFPHGF
TASGHPVGCAIALKAIDVVMNEGLAENVRRLAPRFEERLKHIAERPNI GEYRGIG
FMWALEAVKDKASKTPFDGNLSVSERIANTCTDLGLICSPMGQSVILCPPFILTE
AQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 2)

Example 32

Enzymatic reduction of (*E*)-3-formyl-5-methylhex-2-enoic acid to 3-formyl-5-methylhexanoic acid and *in situ* conversion to pregabalin with ω -transaminase



Reduction of (*E*)-3-formyl-5-methylhex-3-enoic acid to 3-formyl-5-methylhexanoic acid with pentaerythritol tetranitrate reductase and *in situ* conversion to pregabalin was evaluated with various ω -transaminases. Recombinant ω -transaminases from *Vibrio fluvialis*, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans* were expressed in *E. coli* as follows: The pET28b ω -transaminase expression constructs were transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB + kanamycin Medium (Novagen, EMD Chemicals, Gibbstown, NJ, USA). Cultures were incubated for 20h at 30°C, and the cells were harvested by centrifugation (4000 x g, 30min, 4°C) and stored at -20°C.

Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH 7.0) with pentaerythritol tetranitrate reductase (40mg wet cells/mL), *Lactobacillus brevis*

alcohol dehydrogenase (32U/mL), NADP⁺ (0.02mM), 2-propanol (3 vol%), pyridoxal phosphate (2 mM), isopropylamine (100mM), (*E*)-3-formyl-5-methylhex-2-enoic acid (20mM) and ω-transaminase (40mg wet cells/mL) from *Vibrio fluvialis*, *Rhodobacter sphaeroides*, or *Paracoccus denitrificans*. After 43 h, reaction samples (0.1mL) were diluted with 0.1mL acetonitrile:water (1:1, v/v). Aliquots (0.1mL) of the diluted reaction samples were treated with saturated aqueous sodium bicarbonate (0.01mL) and Marfey's reagent (*N*-α-(2,4-dinitro-5-fluorophenyl)alaninamide, 0.4mL of 5g/L solution in acetonitrile) at 40°C. After 1h, the derivatization reactions were quenched with 0.1mL 1N aqueous hydrochloric acid. The derivatized reaction samples were analyzed by UPLC (column: BEH C18, 50mm x 2.1mm id, gradient elution: 70% A:30% B to 55% A:45% B in 5min (A = 1% triethylamine (pH 3 with phosphoric acid); B = acetonitrile), flow rate: 0.8mL/min, column temperature: 30°C, detection: 210-400nm) to give the results shown in Table 7.

Table 7

Entry	Enzyme	Accession Number	%Yield Pregabalin	%ee (S)-Pregabalin
1	<i>Vibrio fluvialis</i> ω-transaminase	AEA39183	60.8	42.0
2	<i>Rhodobacter sphaeroides</i> ω-transaminase	YP_001043908	60.4	54.6
3	<i>Paracoccus denitrificans</i> ω-transaminase	YP_917746	33.6	61.5

Example 33

Enzymatic reduction of (*E*)-3-formyl-5-methylhex-2-enoic acid to 3-formyl-5-methylhexanoic acid and in-situ conversion to pregabalin with *V. fluvialis* ω-transaminase



Reduction of (*E*)-3-formyl-5-methylhex-3-enoic acid to 3-formyl-5-methylhexanoic acid with pentaerythritol tetranitrate reductase and in-situ conversion to pregabalin was evaluated with variants of *V. fluvialis* aminotransferase. Variants of *V. fluvialis* ω -transaminase (Accession number AEA39183) were expressed in *E. coli* as follows:

- 5 QuikChange Site-directed Mutagenesis kits from Stratagene (La Jolla, CA, USA) was used to create *V. fluvialis* aminotransferase variants as directed. Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). The pET28b ω -transaminase expression constructs were transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in
10 LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB + kanamycin Medium (Novagen, EMD Chemicals, Gibbstown, NJ, USA). Cultures were incubated for 20h at 30°C, and the cells were harvested by centrifugation (4000 x g, 30min, 4°C) and stored at -20°C.

- Reactions (0.5mL) were carried out at 30°C in potassium phosphate buffer (100mM, pH
15 7.0) with pentaerythritol tetranitrate reductase (40mg wet cells/mL), *Lactobacillus brevis* alcohol dehydrogenase (32U/mL), NADP⁺ (0.1mM), 2-propanol (3 vol%), pyridoxal phosphate (2mM), isopropylamine (300mM), (*E*)-3-formyl-5-methylhex-2-enoic acid (100mM) and *V. fluvialis* ω -transaminase wild-type or variants (40mg wet cells/mL). After 48h, reaction samples (0.02mL) were diluted with 0.18mL acetonitrile:water (1:1,
20 v/v). Aliquots (0.1mL) of the diluted reaction samples were treated with saturated aqueous sodium bicarbonate (0.01mL) and Marfey's reagent (*N*- α -(2,4-dinitro-5-fluorophenyl)alaninamide, 0.4mL of 5 g/L solution in acetonitrile) at 40°C. After 1 h, the derivatization reactions were quenched with 0.1mL 1N aqueous hydrochloric acid. The derivatized reaction samples were analyzed by UPLC (column: BEH C18, 50mm x
25 2.1mm id, gradient elution: 70% A:30% B to 55% A:45% B in 5 min (A = 1% triethylamine (pH 3 with phosphoric acid); B = acetonitrile), flow rate: 0.8mL/min, column temperature: 30°C, detection: 210-400nm) to give the results shown in Table 8.

Table 8

Entry	<i>V. fluvialis</i> transaminase Variant	%Yield Pregabalin	%ee (S)-Pregabalin
1	M294V	64.8	73.7
2	T268M	47.4	77.8
3	P233L	77.3	71

Entry	<i>V. fluvialis</i> transaminase Variant	%Yield Pregabalin	%ee (S)-Pregabalin
4	N166V	38.1	73.1
5	C424A	76.5	70.6
6	L100M	83.7	71.4
7	S283A	68.1	71
8	L417M	68.7	71.8
9	Wild-type	62.7	61.5

Example 34

Alternative variants of *Vibrio fluvialis* ω -transaminase

The following further recombinant variants of *Vibrio fluvialis* ω -transaminase were expressed in *E. coli* as follows: The pET28b ω -transaminase expression constructs were transformed into BL21(DE3) *E. coli* (Stratagene, Agilent Technologies, Santa Clara, CA, USA) as directed and overnight cultures were grown in LB + kanamycin media. The LB cultures were used to inoculate expression cultures grown in Overnight Express Instant TB + kanamycin Medium (Novagen, EMD Chemicals, Gibbstown, NJ, USA). Cultures were incubated for 20 h at 30°C, and the cells were harvested by centrifugation (4000 x g, 30 min, 4°C) and stored at -20°C.

Example 34a: Vfat906

DNA Sequence:

ATGAATAAACCAAAAGCTGGGAAGCGCGTGCTGAACTTACTCTCTGTACGGCTT
 CACTGATATGCCATCTCTGCACgAGCGTGGTACCGTGGTTGTCACCCACGGCGAG
 GGCCCATACATCGTGGACGTCAACGGTCGCCGTTACCTGGACGCAAACTCCGGCC
 TGTACAATATGGTTGCCGGCTTCGACCACAAGGGTCTGATCGACGCAGCAAAGGC
 CCAGTACGAACGCTTCCCGGGTTACCATAGCTTCTTCGGTCGTATGTCTGATCAAA
 CTGTTATGCTGAGCGAGAACTGGTAGAGGTGTCTCCATTCGACAGCGGTCTCGCT
 GTTCTATACTAACTCCGGCTCCGAGGCTAACGATACTATGGTGAAAATGCTGTGGT
 TTCTGCACGCCGCAGAGGGCAAGCCGCAAAAACGCAAAATCCTGACTCGTaacAAC
 GCATACCACGGTGTAAGTCTGTTTCCGCTTCCATGACGGGTCTGCCGTACAACCTC
 TGTATTCGGCCTGCCGCTGCCGGGTTTCGTTACCTGACCTGTCCGCACTATTGG
 CGTTACGGCGAAGAAGGTGAAACCGAAGAGCAGTTTGTTGCTCGTCTGGCCCGCG
 AGCTGGAGGAACTATCCAACGTGAAGGCGCGGACACGATTGCGGGCTTCTTTGC
 TGAGCCGGTCATGGGCGCGGGCGGCGTAATCCCGCCGGCGAAAGGTTACTTCCA

GGCGATCCTGCCGATTCTGCGTAAGTACGACATCCCGGTTATCTCTGATGAAGTTA
TCTGCGGCTTTGGTCGTACCGGTAATACTTGGGGTTGCGTTACCTATGACTTCACC
CCGGATGCGATCATCTCCAGCAAAAATCTGACCGCCGGTTTCTTTCCGGTTGGTG
CTGTGATTCTGGGTCCGGAAGTCTGAGCAAACGCCTGGAAACGGCGATCGAAGCTAT
5 CGAAGAGTTCCCGCACGGCTTTACGGCC_{ggc}GGTCACCCGGTGGGTTGCGCTATC
GCTCTGAAAGCAATCGATGTTGTGATGAATGAGGGTCTGGCAGAGAACGTGCGCC
GCCTGGCACCGCGTTTTGAGGAGCGTCTGAAACACATTGCCGAACGTCCGAACAT
CGGTGAATATCGTGGCATCGGTTTTATGTGGGCACTGGAGGCTGTGAAAGACAAA
GCATCTAAAACCCCATTCGATGGTAATCTGTCTGTGAGC_{aaa}CGTATCGCTAACACC
10 TGT_{cag}GACCTGGGCCTGATCTGTAGC_gCGCTGGGTGAGTCCGTTATCCTGTGCCC
GCCGTTTCATCCTGACCGAGGCGCAAATGGATGAGATGTTTGACAACTGGAGAAG
GCTCTGGACAAAGTCTTTGCGGAGGTGGCGTAA (SEQ ID NO. 11)

Amino acid sequence:

15 MNKPQSWEARAETYSLYGFTDMPSLHERGTVVWTHGEGPYIVDVNGRRYLDANSGLY
NMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVFYT
NSGSEANDTMVKMLWFLHAAEGKPQKRKILTRNNAYHGVTAVSASMTGLPYNSVFGL
PLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGAG
GVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAIISKNLTAG
20 FFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENVR
RLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQD
LGLICSALGQSVILCPPFILTEAQMDMFDKLEKALDKVFAEVA (SEQ ID NO. 3)

Example 34b: Vfat999

25 DNA Sequence:

ATGAATAAACCAACAAAGCTGGGAAGCGCGTGCTGAACTTACTCTCTGTACGGCTT
CACTGATATGCCATCTCTGCACGAGCGTGGTACCGTGGTTGTCACCCACGGCGAG
GGCCCATACGTGGTGGACGTCAACGGTCGCCGTTACCTGGACGCAAACTCCGGC
CTGTACAATATGGTTGCCGGCTTCGACCACAAGGGTCTGATCGACGCAGCAAAGG
30 CCCAGTACGAACGCTTCCCGGGTTACCATAGCTTCTTCGGTCGTATGTCTGATCAA
ACTGTTATGCTGAGCGAGAACTGGTAGAGGTGTCTCCATTCGACAGCGGTCGCG
TGTTCTATACTAACTCCGGCTCCGAGGCTAACGATACTATGGTGAAAATGCTGTGG
TTTCTGCACGCCGCAGAGGGCAAGCCGCAAAAACGCAAAATCCTGACTCGTCAAA
ACGCATACCACGGTGTAAGTCTGTTTCCGCTTCCATGACGGGTCTGCCGCACAA
35 CTCTGTATTCGGCCTGCCGCTGCCGGGTTTCGTTACCTGACCTGTCCGCACTATT

GGCGTTACGGCGAAGAAGGTGAAACCGAAGAGCAGTTTGTGCTCGTCTGGCCCG
 CGAGCTGGAGGAACTATCCAACGTGAAGGCGCGGACACGATTGCGGGCTTCTTT
 GCTGAGCCGGTCATGGGCGCGGGCGGGCGTAATCCCGCCGGCGAAAGGTTACTTC
 CAGGCGATCCTGCCGATTCTGCGTAAGTACGACATCCCGGTTATCTCTGATGAAGT
 5 TATCTGCGGCTTTGGTCGTACCGGTAATACTTGGGGTTGCGTTACCTATGACTTCA
 CCGCGGATGCGATCATCTCCAGCAAAAATCTGACCGCCGGTTTCTTTCCGGTTGGT
 GCTGTGATTCTGGGTCCGGAACCTGAGCAAACGCCTGGAAACGGCGATCGAAGCTA
 TCGAAGAGTTCCCGCACGGCTTTACGGCCGGCGGTCAACCGGTGGGTGCGCTA
 TCGCTCTGAAAGCAATCGATGTTGTGATGAATGAGGGTCTGGCAGAGAACGTGCG
 10 CCGCCTGGCACC GCGTTTTGAGGAGCGTCTGAAACACATTGCCGAACGTCCGAAC
 ATCGGTGAATATCGTGGCATCGGTTTTATGTGGGCACTGGAGGCTGTGAAAGACA
 AAGCATCTAAAACCCCATTCGATGGTAATCTGTCTGTGAGCAAACGTATCGCTAAC
 ACCTGTCAGGACCTGGGCCTGATCTGTAGCGCGCTGGGTCAAGTCCGTTATCCTGA
 GCCCGCCGTTTCATCCTGACCGAGGCGCAAATGGATGAGATGTTTGACAAACTGGA
 15 GAAGGCTCTGGACAAAGTCTTTGCGGAGGTGGCGTAA (SEQ ID NO. 12)

Amino acid sequence:

MNKPQSWEARAETYSLYGFTDMPSLHERGTVVWTHGEGPYVVDVNGRRYLDANGL
 YNMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVfy
 20 TNSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGLPHNSVFG
 LPLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
 GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAISSKNLTA
 GFFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
 RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
 25 DLGLICSALGQSVILSPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 4)

Example 34c: Vfat1010

DNA Sequence:

ATGAATAAACCAACAAAGCTGGGAAGCGCGTGCTGAAACTTACTCTCTGTACGGCTT
 30 CACTGATATGCCATCTCTGCACGAGCGTGGTACCGTGGTTGTCACCCACGGCGAG
 GGCCCATACATCGTGGACGTCAACGGTCGCCGTTACCTGGACGCAAACTCCGGCC
 TGTACAATATGGTTGCCGGCTTCGACCACAAGGGTCTGATCGACGCAGCAAAGGC
 CCAGTACGAACGCTTCCCGGGTTACCATAGCTTCTTCGGTCGTATGTCTGATCAAA
 CTGTTATGCTGAGCGAGAACTGGTAGAGGTGTCTCCATTCGACAGCGGTGCGGT
 35 GTTCTATACTAACTCCGGCTCCGAGGCTAACGATACTATGGTGAAAATGCTGTGGT

TTCTGCACGCCGCAGAGGGCAAGCCGCAAAAACGCAAAATCCTGACTCGTCAAAA
CGCATACCACGGTGTAACTGCTGTTTCCGCTTCCATGACGGGTATGCCGCACAAC
TCTGTATTCGGCCTGCCGCTGCCGGGTTTCGTTACCTGACCTGTCCGCACTATTG
GCGTTACGGCGAAGAAGGTGAAACCGAAGAGCAGTTTGTTGCTCGTCTGGCCCGC
5 GAGCTGGAGGAACTATCCAACGTGAAGGCGCGGACACGATTGCGGGCTTCTTTG
CTGAGCCGGTCATGGGCGCGGGCGGCGTAATCCCGCCGGCGAAAGGTTACTTCC
AGGCGATCCTGCCGATTCTGCGTAAGTACGACATCCCGGTTATCTCTGATGAAGTT
ATCTGCGGCTTTGGTCGTACCGGTAATACTTGGGGTTGCGTTACCTATGACTTCAC
CCCGGATGCGATCATCTCCAGCAAAAATCTGACCGCCGGTTTCTTTCCGGTTGGT
10 GCTGTGATTCTGGGTCCGGCACTGAGCAAACGCCTGGAAACGGCGATCGAAGCTA
TCGAAGAGTTCCCGCACGGCTTTACGGCCGGCGGTCACCCGGTGGGTGCGCTA
TCGCTCTGAAAGCAATCGATGTTGTGATGAATGAGGGTCTGGCAGAGAACGTGCG
CCGCCTGGCACCGCGTTTTGAGGAGCGTCTGAAACACATTGCCGAACGTCCGAAC
ATCGGTGAATATCGTGGCATCGGTTTTATGTGGGCACTGGAGGCTGTGAAAGACA
15 AAGCATCTAAAACCCCATTCGATGGTAATCTGTCTGTGAGCAAACGTATCGCTAAC
ACCTGTCAGGACCTGGGCCTGATCTGTAGCGCGATGGGTCAGTCCGTTATCCTGA
GCCCCCGTTTCATCCTGACCGAGGCGCAAATGGATGAGATGTTTGACAAACTGGA
GAAGGCTCTGGACAAAGTCTTTGCGGAGGTGGCGTAA (SEQ ID NO. 13)

20 Amino acid sequence:

MNKPQSWEARAETYSLYGFTDMPSLHERGTVVWTHGEGPYIVDVNGRRYLDANSGLY
NMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVFT
NSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGMPHNSVFG
LPLPGFVHLTCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
25 GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAIISSKNLTA
GFFPVGAVILGPALSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
DLGLICSAMGQSVILSPPFILTEAQMDMFDKLEKALDKVFAEVA (SEQ ID NO. 5)

30 **Example 34d: Vfat1020**

DNA Sequence:

ATGAATAAACCAACAAAGCTGGGAAGCGCGTGCTGAACTTACTCTCTGTACGGCTT
CACTGATATGCCATCTCTGCACGAGCGTGGTACCGTGGTTGTACCCACGGCGAG
GGCCCATACGTGGTGGACGTCAACGGTCGCCGTTACCTGGACGCAAACTCCGGC
35 CTGTACAATATGGTTGCCGGCTTCGACCACAAGGGTCTGATCGACGCAGCAAAGG

CCCAGTACGAACGCTTCCCGGGTTACCATAGCTTCTTCGGTCGTATGTCTGATCAA
 ACTGTTATGCTGAGCGAGAACTGGTAGAGGTGTCTCCATTGACAGCGGTGCGG
 TGTTCTATACTAACTCCGGCTCCGAGGCTAACGATACTATGGTGAAAATGCTGTGG
 TTTCTGCACGCCGAGAGGGCAAGCCGCAAAAACGCAAAATCCTGACTCGTCAAA
 5 ACGCATACCACGGTGTAAGTGTGTTTCCGCTTCCATGACGGGTCTGCCGCACAA
 CTCTGTATTCGGCCTGCCGCTGCCGGGTTTCGTTACCTGGGTTGTCCGCACTATT
 GCGGTTACGGCGAAGAAGGTGAAACCGAAGAGCAGTTTGTGCTCGTCTGGCCCCG
 CGAGCTGGAGGAACTATCCAACGTGAAGGCGCGGACACGATTGCGGGCTTCTTT
 GCTGAGCCGGTCATGGGCGCGGGCGGCGTAATCCCGCCGGCGAAAGGTTACTTC
 10 CAGGCGATCCTGCCGATTCTGCGTAAGTACGACATCCCGGTTATCTCTGATGAAGT
 TATCTGCGGCTTTGGTCGTACCGGTAATACTTGGGGTTGCGTTACCTATGACTTCA
 CCCC GGATGCGATCATCTCCAGCAAAAATCTGACCGCCGGTTTCTTTCCGGTTGGT
 GCTGTGATTCTGGGTCCGGAAGTGTGAGCAAACGCCTGGAAACGGCGATCGAAGCTA
 TCGAAGAGTTCCCGCACGGCTTTACGGCCGGCGGTCACCCGGTGGGTTGCGCTA
 15 TCGCTCTGAAAGCAATCGATGTTGTGATGAATGAGGGTCTGGCAGAGAACGTGCG
 CCGCCTGGCACCGCGTTTTGAGGAGCGTCTGAAACACATTGCCGAACGTCCGAAC
 ATCGGTGAATATCGTGGCATCGGTTTTATGTGGGCACTGGAGGCTGTGAAAGACA
 AAGCATCTAAAACCCCATTCGATGGTAATCTGTCTGTGAGCAAACGTATCGCTAAC
 ACCTGTCAGGACCTGGGCCTGATCTGTAGCGCGATGGGTCAGTCCGTTATCCTGA
 20 GCCCGCCGTTTCATCCTGACCGAGGCGCAAATGGATGAGATGTTTGACAAACTGGA
 GAAGGCTCTGGACAAAGTCTTTGCGGAGGTGGCGTAA (SEQ ID NO. 14)

Amino acid sequence:

MNKPQSWEARAETYSLYGFTDMPSLHERGTVVWTHGEGPYVVDVNGRRYLDANGL
 25 YNMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVfy
 TNSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGLPHNSVFG
 LPLPGFVHLGCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGA
 GGVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAISSKNLTA
 GFFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENV
 30 RRLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQ
 DLGLICAAMGQSVILSPPFILTEAQMDMFDKLEKALDKVFAEVA (SEQ ID NO. 6)

Example 34e: Vfat1030

DNA Sequence:

ATGAATAAACCAACAAAGCTGGGAAGCGCGTGCTGAACTTACTCTCTGTACGGCTT
CACTGATATGCCATCTCTGCACGAGCGTGGTACCGTGGTTGTCACCCACGGCGAG
GGCCCATACATCGTGGACGTCCACGGTCGCCGTTACCTGGACGCAAACTCCGGC
CTGTACAATATGGTTGCCGGCTTCGACCACAAGGGTCTGATCGACGCAGCAAAGG
5 CCCAGTACGAACGCTTCCCGGGTTACCATAGCTTCTTCGGTCGTATGTCTGATCAA
ACTGTTATGCTGAGCGAGAACTGGTAGAGGTGTCTCCATTGACAGCGGTGCGG
TGTTCTATACTAACTCCGGCTCCGAGGCTAACGATACTATGGTGAAAATGCTGTGG
TTTCTGCACGCCGCGAGAGGGCAAGCCGCAAAAACGCAAAATCCTGACTCGTCAA
ACGCATACCACGGTGTAAGTGTGTTTCCGCTTCCATGACGGGTCTGCCGCACAA
10 CTCTGTATTCCGGCCTGCCGCTGCCGGGTTTCGTTACCTGAGCTGTCCGCACTATT
GGCGTTACGGCGAAGAAGGTGAAACCGAAGAGCAGTTTGTTGCTCGTCTGGCCCCG
CGAGCTGGAGGAACTATCCAACGTGAAGGCGCGGACACGATTGCGGGCTTCTTT
GCTGAGCCGGTCATGGGCGCGGGCGGGCGTAATCCCGCCGGCGAAAGGTTACTTC
CAGGCGATCCTGCCGATTCTGCGTAAGTACGACATCCCGGTTATCTCTGATGAAGT
15 TATCTGCGGCTTTGGTCGTACCGGTAATACTTGGGGTTGCGTTACCTATGACTTCA
CCCCGGATGCGATCATCTCCAGCAAAAATCTGACCGCCGGTTTCTTTCCGGTTGGT
GCTGTGATTCTGGGTCCGGAAGTGAAGCAACGCCTGGAAACGGCGATCGAAGCTA
TCGAAGAGTTCCCGCACGGCTTTACGGCCGGCGGTCACCCGGTGGGTGCGCTA
TCGCTCTGAAAGCAATCGATGTTGTGATGAATGAGGGTCTGGCAGAGAACGTGCG
20 CCGCCTGGCACCGCGTTTTGAGGAGCGTCTGAAACACATTGCCGAACGTCCGAAC
ATCGGTGAATATCGTGGCATCGGTTTTATGTGGGCACTGGAGGCTGTGAAAGACA
AAGCATCTAAAACCCCATTCGATGGTAATCTGTCTGTGAGCAAACGTATCGCTAAC
ACCTGTCAGGACCTGGGCCTGATCTGTAGCGCGATGGGTCAGTCCGTTATCCTGA
GCCCGCCGTTTCATCCTGACCGAGGCGCAAATGGATGAGATGTTTGACAAACTGGA
25 GAAGGCTCTGGACAAAGTCTTTGCGGAGGTGGCGTAA (SEQ ID NO. 15)

Amino acid sequence:

MNKPQSWEARAETYSLYGFTDMPSLHERGTVVWTHGEGPYIVDVHGRRYLDANGLY
NMVAGFDHKGLIDAAKAQYERFPGYHSFFGRMSDQTVMLSEKLVEVSPFDSGRVFYT
30 NSGSEANDTMVKMLWFLHAAEGKPQKRKILTRQNAYHGVTAVSASMTGLPHNSVFGL
PLPGFVHLSCPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGAG
GVIPPAKGYFQAILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDAIISKNLTAG
FFPVGAVILGPELSKRLETAIEAIEEFPHGFTAGGHPVGCAIALKAIDVVMNEGLAENVR
RLAPRFEERLKHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSKRIANTCQD
35 LGLICSAMGQSVILSPPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO. 7)

Example 35**Biotransformation of 3-isobutylidene-2-oxopentanedioic acid to Pregabalin**

- 5 The genes for the *Pseudomonas putida* benzoylformate decarboxylase, *Enterobacter cloacae* pentaerythritol tetranitrate reductase, *Lactobacillus brevis* alcohol dehydrogenase, and *Vibrio fluvialis* ω -transaminase were cloned into expression vector pDSTRC52 (Pfizer Inc., USA) and put into the expression strain *E. coli* BDG62 (Pfizer Inc., USA). The culture was grown and enzyme production was induced as described in
- 10 Example 22. Enzyme expression was determined by polyacrylamide gel electrophoresis using Novex gels and stains (Invitrogen Corporation Carlsbad, California).

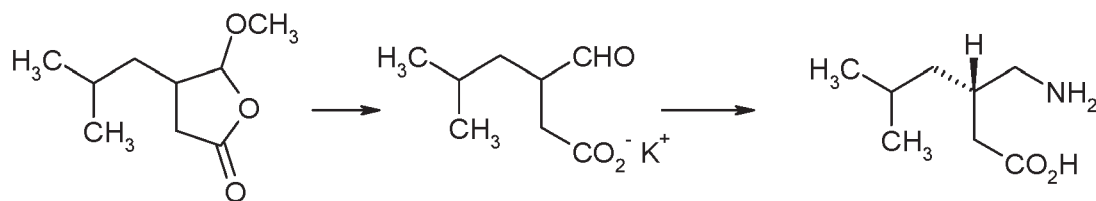
- Reactions (1.0mL) were carried out at 40°C in phosphate buffer (100mM, pH 6.4) with
- 15 *P. putida* decarboxylase, *V. fluvialis* transaminase, *L. brevis* alcohol dehydrogenase, *E. cloacae* pentaerythritol tetranitrate reductase, and NADP (0.1mM) in one cell. 200 μ L 1M 3-isobutylidene-2-oxopentanedioic acid, 100 μ L 1mM ThDp + 25mM MgSO₄, 40 μ L 50mM PLP, 30 μ L isopropanol, 250 μ L 2M isopropylamine. Adjust pH to 6.4 for 24 hours, then adjust pH to 6.8 for additional 24 hours. An aliquot (0.5mL) of the reaction was
- 20 treated with 1M aqueous sodium bicarbonate (0.05mL) and Marfey's reagent (*N*- α -(2,4-dinitro-5-fluorophenyl)alaninamide, 0.5mL of 5g/L solution in acetonitrile) at 40°C. After 1 h, the derivatization reactions were quenched with 0.05mL 1N aqueous hydrochloric acid. The derivatized reaction samples were filtered and analyzed by UPLC (column: Agilent Eclipse Plus C18 column (100mm x 3.0mm, 1.8 μ m) eluted with 0.1%
- 25 trifluoroacetic acid in water:acetonitrile (60:40, v/v) at 1.3mL/min. The column was maintained at 30°C and the effluent was monitored at 340nm and ES⁺ mass spectroscopy. The reaction yielded a small amount of Pregabalin of which 82% was the desired S-isomer.

Example 36**Biotransformation of 3-(2-methylpropyl)-2-oxopentanedioic acid to Pregabalin**

The genes for the *Pseudomonas putida* benzoylformate decarboxylase and *Vibrio*
 5 *fluvialis* ω -transaminase were cloned into expression vector pDSTRC52 (Pfizer Inc.,
 USA) and put into the expression strain *E. coli* BDG62 (Pfizer Inc., USA). The culture
 was grown and enzyme production was induced as described in Example 22. Enzyme
 expression was determined by polyacrylamide gel electrophoresis using Novex gels and
 stains (Invitrogen Corporation Carlsbad, California).

10

Reactions (2.0mL) were carried out at 40°C in phosphate buffer (100mM, pH 6.4) with
 500 μ L of culture (42.5mg dry cell weight) from a 24 hour fermentation tanks with *P.*
putida decarboxylase and *V. fluvialis* ω -transaminase cloned in one plasmid, 400 μ L
 0.5M 3-(2-methylpropyl)-2-oxopentanedioic acid, 200 μ L 10 x ThDP (final 0.1mM) and
 15 MgSO₄ (final 2.5mM), 80 μ L 50mM PLP (final 2mM), 300 μ L 2M isopropylamine (final
 0.3M). Adjust pH to 6.4 and incubate at 45°C for 24 hours, then adjust pH to 6.8 for
 additional 24 hours. An aliquot (0.5mL) of the reaction was treated with 1M aqueous
 sodium bicarbonate (0.05mL) and Marfey's reagent (*N*- α -(2,4-dinitro-5-
 fluorophenyl)alaninamide, 0.5mL of 5g/L solution in acetonitrile) at 40°C. After 1h, the
 20 derivatization reactions were quenched with 0.05mL 1N aqueous hydrochloric acid.
 The derivatized reaction samples were filtered and analyzed by UPLC (column: Agilent
 Eclipse Plus C18 column (100mm x 3.0mm, 1.8 μ m) eluted with 0.1% trifluoroacetic acid
 in water:acetonitrile (60:40, v/v) at 1.3mL/min. The column was maintained at 30°C and
 the effluent was monitored at 340nm and ES⁺ mass spectroscopy. The reaction yielded
 25 100 μ g/mL of Pregabalin of which 65% was the desired S-isomer.

Example 37**Preparation of (S)-Pregabalin via hydrolysis of 5-methoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one and enzymatic transamination**

5 5-Methoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one (2.58g, 15mmol, see example 13) was suspended in DIW (5.2g) and cooled in an ice/water bath. Aq KOH (50% w/w, 1.77g, 1.05eq) was added dropwise *via* syringe over 5mins. The reaction was removed from the ice/water bath and stirred at rt for 90mins. The pH was adjusted to 7.0 using formic acid. The reaction mixture was then used as feedstock in the

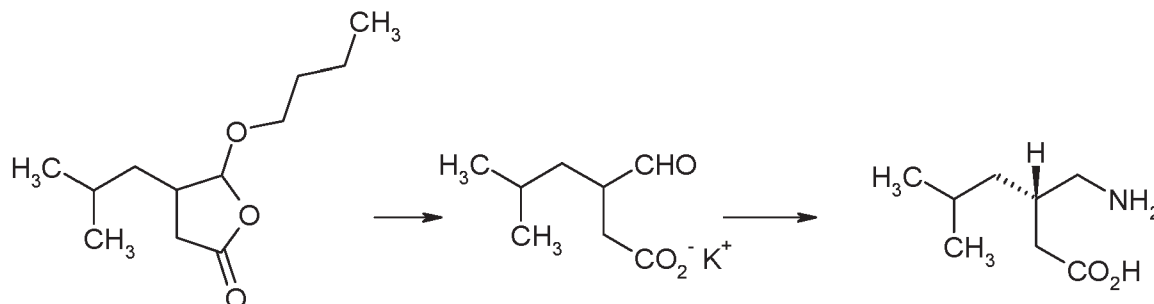
10 subsequent transaminase reaction.

Transaminase enzyme solution (12.5g), PLP (35mg), DIW (15mL) and 4.0M isopropylamine.HCl aq soln (7.5mL, 30mmol) were charged to a 100mL flask and warmed to 45°C. The hydrolysis reaction was added in one portion followed by DIW (6 mL, used as a vessel rinse). The pH of the reaction was adjusted to 7.25 using neat

15 isopropylamine and the reaction was stirred at 45°C until reaction completion. The reaction mixture was then heated to an internal temperature of 55°C and the pH adjusted to 4.0 using 95% formic acid. Darco carbon (125mg) was added and the mixture was allowed to cool to room temperature before cooling on ice/water for 20 minutes. The mixture was then filtered through Whatman paper no. 3. The filtrate was

20 concentrated to 1/3 of its weight and then heated to 55°C. The pH of the solution was then adjusted to pH 7.5 using 50% KOH after which the solution was cooled to ambient and then to 0-5°C in an ice/water bath. Precipitation of product was observed in the cooldown. The slurry was filtered and washed with DIW/EtOH (10mL, 1:1, 0°C). The white precipitate was dried for 12 hours in a vacuum oven (45°C) to yield (S)-

25 Pregabalin in 61% yield, 98.6% w/w purity and 99. 8% ee (preferred S-isomer).

Example 38**Preparation of (S)-Pregabalin via hydrolysis of 5-butoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one and enzymatic transamination.**

5 5-Butoxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one (3.21g, 15mmol, see example 13C) was suspended in DIW (8.5g) and cooled in an ice/water bath. Aq KOH (50% w/w, 2.02g, 1.2eq) was added dropwise via syringe over 5mins. The reaction was removed from the ice/water bath and stirred at rt for 90mins. The reaction pH was then adjusted to pH 7.0 using formic acid, and the reaction mixture was then used as

10 feedstock in the subsequent transaminase reaction.

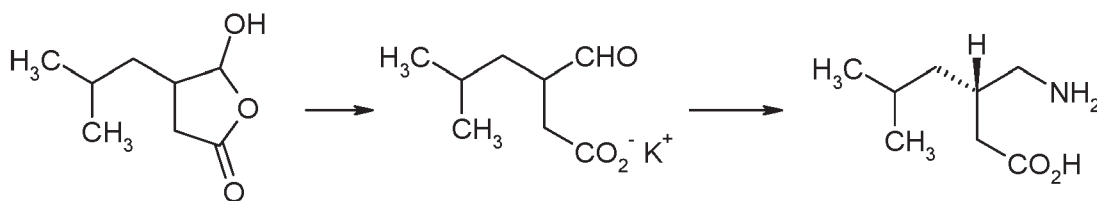
Transaminase enzyme solution (12.5g), PLP (35mg), DIW (15mL) and 4.0M isopropylamine.HCl solution (7.5mL, 30mmol) were charged to a 100mL flask and warmed to 45°C. The hydrolysis reaction (see above) was added in one portion followed by DIW (6mL, used as a vessel rinse). The pH of the reaction was adjusted to

15 7.25 using neat isopropylamine and the reaction was stirred at 45°C.

The reaction mixture was heated to an internal temperature of 55°C and pH adjusted to 4.0 using 95% formic acid. Darco carbon (125mg) was added and the mixture was allowed to cool to room temperature before being cooled on ice/water for 20 minutes. The mixture was then filtered through Whatman paper no 3. The filtrate was

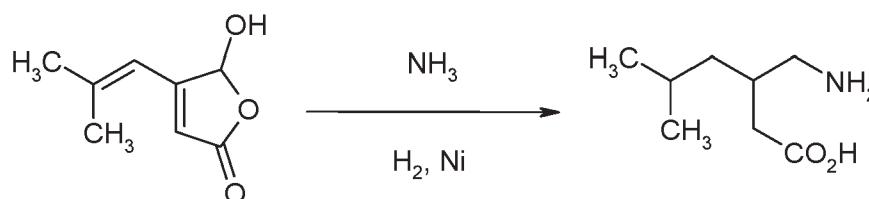
20 concentrated to 1/3 of its weight then heated to 55°C. The pH of the solution was then adjusted to pH 7.5 using 50% KOH after which the solution was cooled to ambient and then to 0-5°C in an ice/water bath. Precipitation of product was observed in the cooldown. The slurry was filtered and washed with DIW/EtOH (10mL, 1:1, 0°C). The white precipitate was dried for 12 hours in a vacuum oven (45°C) to yield (S)-

25 Pregabalin in 51% yield, 98.4% w/w purity and 99.9% e.e preferred S-isomer.

Example 39**Preparation of (S)-Pregabalin from 5-hydroxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one via enzymatic transamination**

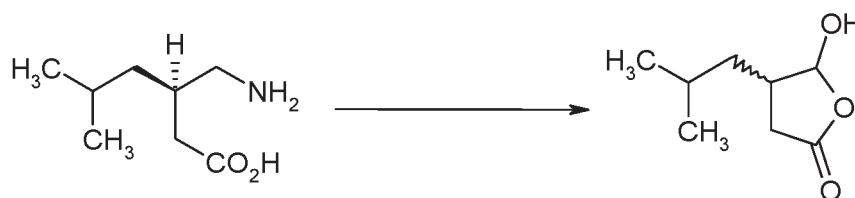
5 5-Hydroxy-4-(2-methylpropyl)-dihydrofuran-2(3H)-one (2.0g, 12.5mmol) was suspended in DIW (8.5g) and cooled in an ice/water bath. Potassium carbonate (0.863g, 6.3mmol) was added portion wise over the course of 5mins. The reaction was removed from the ice/water bath and stirred at rt for 90mins. The pH was adjusted to 7.0 using formic acid and the reaction mixture was then used as feedstock in the subsequent transaminase reaction.

Transaminase enzyme solution (10.4g), PLP (30mg), DIW (12.5mL) and aq 4.0M isopropylamine.HCl aq soln (6.3mL, 30mmol) were charged to a 100mL flask and warmed to 45°C. The hydrolysis reaction was added in one portion followed by DIW (5mL, used as a vessel rinse). The pH of the reaction was adjusted to 7.25 using neat isopropylamine and the reaction was stirred at 45°C. The reaction mixture was heated to an internal temperature of 55°C and adjusted to pH 4.0 using 95% formic acid. Darco carbon (125mg) was added and the mixture was allowed to cool to room temperature before being cooled on ice/water for 20 minutes. The mixture was then filtered through Whatman paper no 3. The filtrate was concentrated to 1/3 of its weight then heated to 55°C. The pH of the solution was then adjusted to pH 7.5 using 50% KOH after which the solution was cooled to ambient and then to 0-5°C in an ice/water bath. Precipitation of product was observed in the cooldown. The slurry was filtered and washed with DIW/EtOH (10mL, 1:1, 0°C). The white precipitate was dried for 12 hours in a vacuum oven (45°C) to yield (S)-Pregabalin in 61% yield, 98.3% w/w purity and 99.9% e.e of the preferred S-isomer.

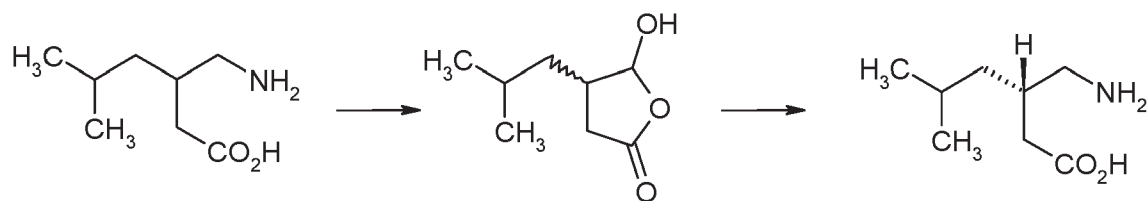
Example 40**Preparation of (R/S)-3-aminomethyl-5-methylhexanoic acid via Reductive amination of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one**

- 5 A 0.01M solution of 5-hydroxy-4-(2-methylprop-1-en-1-yl)furan-2(5H)-one in 30% aqueous ammonia solution was hydrogenated (10 bar, ambient temperature) for 48h in the presence of 10mol% Raney Nickel catalyst. The catalyst was filtered and the solution concentrated to leave a solid. The product was isolated by addition of hydrochloric acid to a methanol suspension and found to be pure 3-aminomethyl-5-methylhexanoic acid hydrochloride.
- 10

The use of palladium as a catalyst gave mixtures of 3-aminomethyl-5-methylhexanoic acid and the corresponding secondary amine, 3-[(2-carboxymethyl-4-methylpentylamino)-methyl]-5-methyl-hexanoic acid.

Example 41**Conversion of R-3-aminomethyl-5-methylhexanoic acid to 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone using a transaminase**

- A solution of R-3-aminomethyl-5-methylhexanoic acid in D.I. water at pH 7.5/ 45⁰C is stirred with a transaminase enzyme lysate, or whole cell preparation containing pyridoxal phosphate (PLP) and acetone. The isopropylamine produced is removed via a nitrogen sweep. Analysis of the solution after 24h show 3-aminomethyl-5-methylhexanoic acid with a lower e.e. and the presence of compound 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone. The lowering of the chiral purity of the 5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone is due to the selective conversion of (4S)-5-hydroxy-4-(2-methylpropyl)-3,4-dihydro-5H-2-furanone to S-pregabalin.
- 20
- 25

Example 42**De-racemisation of *rac*-pregabalin using a transaminase**

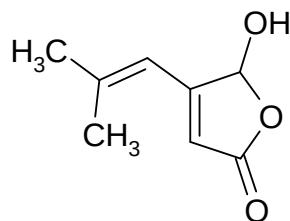
- 5 A solution of racemic pregabalin in DIW at pH 7.5 is treated with a suitable transaminase lysate. PLP is added and the reaction progressed for 12h at 45⁰C with a nitrogen sweep. Then, isopropylamine is added and the reaction is continued for a further 12h. The product is isolated as in Example 38 to afford enantiomerically enriched pregabalin.

10

It is also possible to effect the transformations exemplified in Examples 41 and 42 using a suitable amine oxidase/ imine reductase enzyme system with a co-factor such as NADP.

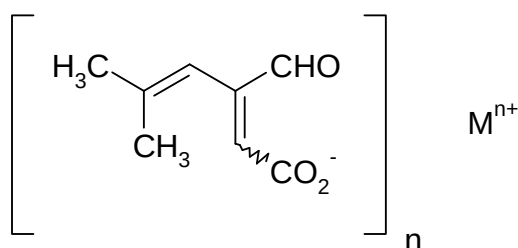
CLAIMS:

1. The compound 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone according to formula (VI^A)



(VI^A)

2. A compound of formula (IX)

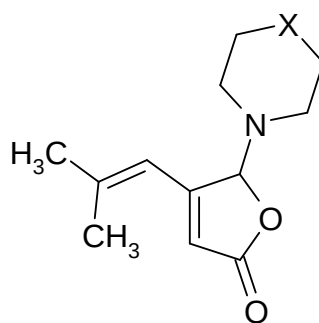


(IX)

wherein:

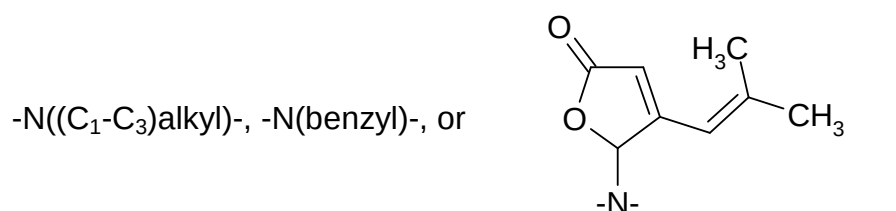
n is 1 and M⁺ is selected from Li⁺, Na⁺, K⁺, Rb⁺, NH₄⁺, ((C₁-C₃)alkyl)NH₃⁺, ((C₁-C₃)alkyl)₂NH₂⁺, ((C₁-C₃)alkyl)₃NH⁺ and ((C₁-C₃)alkyl)₄N⁺; or
n is 2 and M²⁺ is selected from Mg²⁺, Ca²⁺ and Zn²⁺.

3. The compound according to claim 2 wherein n is 1 and M⁺ is selected from NH₄⁺ and ((C₁-C₃)alkyl)NH₃⁺.
4. The compound according to claim 2 wherein n is 1 and M⁺ is selected from Li⁺, Na⁺ and K⁺.
5. A compound of formula (VII)



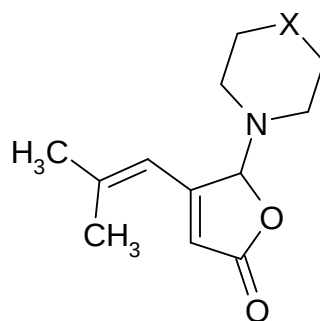
(VII)

wherein –X- represents a single bond, –CH₂–, –O–, –NH–,



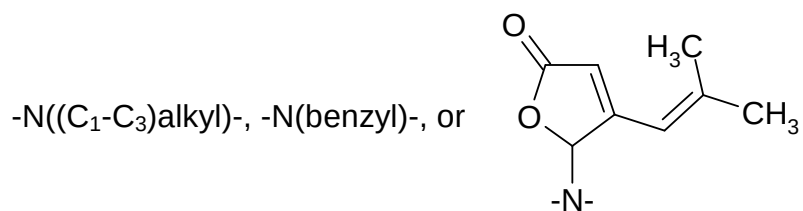
6. The compound according to claim 5 selected from:
 4-(2-methylpropenyl)-5-pyrrolidin-1-yl-5*H*-furan-2-one;
 4-(2-methylpropenyl)-5-piperidin-1-yl-5*H*-furan-2-one;
 4-(2-methylpropenyl)-5-morpholin-4-yl-5*H*-furan-2-one; and
 1,4-*bis*-(4-(2-methylpropenyl)-5*H*-furan-2-on-5-yl)piperazine.

7. A process for the manufacture of the compound of formula (VI^A) according to claim 1 comprising the step of treating a compound of formula (VII)



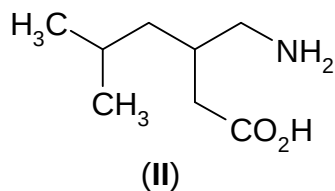
(VII)

wherein –X- represents a single bond, –CH₂–, –O–, –NH–,



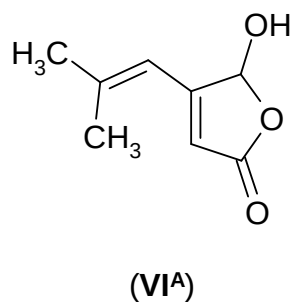
with water in the presence of an acid catalyst.

8. A process for the manufacture of 3-aminomethyl-5-methylhexanoic acid (**II**)



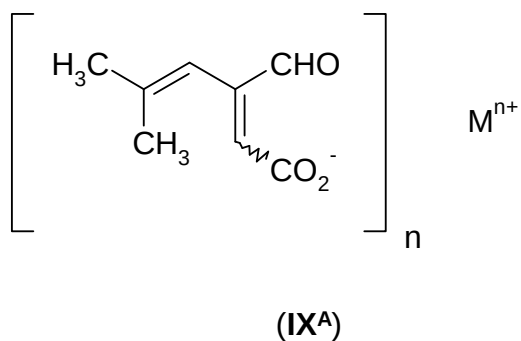
or a pharmaceutically acceptable salt thereof, comprising the steps:

- (a) preparing 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**)



by the process according to claim 7;

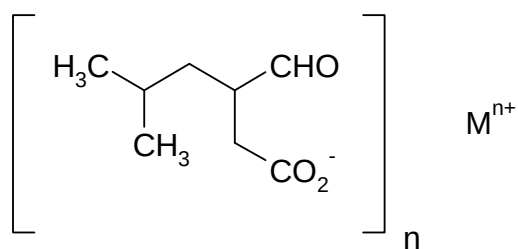
- (b) treating the 5-hydroxy-4-(2-methyl-1-propenyl)-5*H*-2-furanone (**VI^A**) with ammonia or a mono-(C₁-C₃)alkylamine to form a salt of formula (**IX^A**)



wherein:

n is 1 and M⁺ is selected from NH₄⁺ and ((C₁-C₃)alkyl)NH₃⁺;

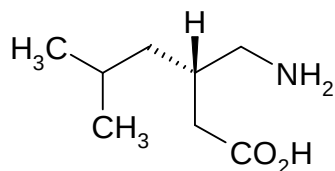
- (c) hydrogenating the salt of formula (**IX^A**) to obtain a salt of formula (**X^A**)



(X^A) ; and

(d) treating the salt of formula (X^A) with a transaminase or an amine oxidase/imine reductase enzyme to provide 3-aminomethyl-5-methylhexanoic acid (II).

9. The process according to claim 8 wherein the 3-aminomethyl-5-methylhexanoic acid (II) is (S)-3-aminomethyl-5-methylhexanoic acid ((S)-II)



((S)-II)

wherein said (S)-3-aminomethyl-5-methylhexanoic acid has an enantiomeric excess of at least 80%.