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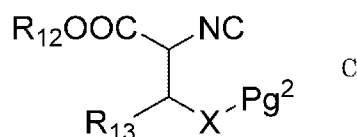
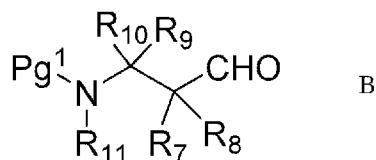
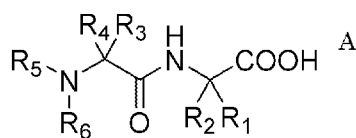
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(54) Title: TUBULYSIN DERIVATIVES AND METHODS FOR PREPARING THE SAME



(57) Abstract: The invention relates to novel means and methods for the synthesis of tubulysin and derivatives thereof, which find their use e.g. as cytotoxic agents in targeted drug delivery. Provided is a method for preparing a tubulysin derivative, comprising reacting compounds A, B and C in a 3-component Passerini reaction, wherein compound A is a carboxylic acid according to the general formula (A); wherein compound B is an aldehyde according to the general formula (B); and wherein compound C is an isocyanide according to the general formula (C).



Title: Tubulysin derivatives and methods for preparing the same.

5 The invention relates to the field of medicinal chemistry. In particular, it relates to novel means and methods for the synthesis of tubulysin and derivatives thereof, for use as cytotoxic agents e.g. in targeted drug delivery.

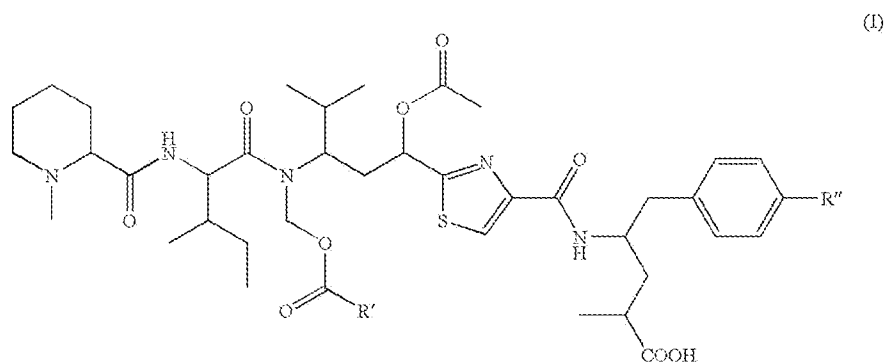
 Antibody-drug conjugates or ADCs are an important class of highly
10 potent biopharmaceutical drugs designed as a targeted therapy for the treatment of cancer. Unlike chemotherapy, ADCs are intended to target and kill only the cancer cells and spare healthy cells. ADCs are complex molecules composed of an antibody linked to a biologically active cytotoxic (anticancer) payload or drug. Antibody-drug conjugates are examples of
15 bioconjugates and immunoconjugates. By combining the unique targeting capabilities of monoclonal antibodies with the cancer-killing ability of cytotoxic drugs, antibody-drug conjugates are designed to allow sensitive discrimination between healthy and diseased tissue. This means that, in contrast to traditional chemotherapeutic agents, antibody-drug conjugates
20 should selectively target and attack the cancer cell so that healthy cells are less severely affected.

 A stable link between the antibody and cytotoxic agent is a crucial aspect of an ADC. A highly stable ADC linker will ensure that less of the cytotoxic payload falls off in circulation, driving an improved safety profile,
25 and will also ensure that more of the payload arrives at the cancer cell, driving enhanced efficacy. Linkers are based on chemical motifs including disulfides, hydrazones or peptides (cleavable), or thioethers (noncleavable) and control the distribution and delivery of the cytotoxic agent to the target cell.

Highly toxic small molecules or natural products have recently found important commercial applications in ADCs. Currently, dolastatine, maytansin and other natural product derivatives are coupled to antibodies to increase their efficacy. Several of such compounds are marketed already and multiple are in clinical trials. (Maturing antibody–drug conjugate pipeline hits 30 Asher Mullard, Nature Reviews Drug Discovery 12, 329–332 (2013) doi:10.1038/nrd4009).

However, current ADC suffer from a number of drawbacks. For example, there is only a limited number of useful drugs, and some induce toxic side effects based on the toxins, the inappropriate linkers, and/or the inappropriate targets/antibodies.

The tubulysins, first isolated by the Hofle/Reichenbach group from myxobacterial cultures are exceptionally potent cell-growth inhibitors that act by inhibiting tubulin polymerisation and thereby induce apoptosis. See Sasse et al. J. Antibiot. 2000, 53, 879-885; WO98/13375. These compounds show high cytotoxicity in the low picomolar IC_{50} in a panel of cancer cell lines and are thus of interest as potential anticancer therapeutics. Tubulysins (I) are tetrapeptides, containing three unusual amino acids; thus, the total synthesis poses a considerable challenge to organic chemists.



20

- Tubulysin A: $R' = CH_2CH(CH_3)_2$; $R'' = OH$
- Tubulysin B: $R' = CH_2CH_2CH_3$; $R'' = OH$
- Tubulysin C: $R' = CH_2CH_3$; $R'' = OH$

- Tubulysin D: $R'=\text{CH}_2\text{CH}(\text{CH}_3)_2$; $R''=\text{H}$
- Tubulysin E: $R'=\text{CH}_2\text{CH}_2\text{CH}_3$; $R''=\text{H}$
- Tubulysin F: $R'=\text{CH}_2\text{CH}_3$; $R''=\text{H}$

The extremely high cytotoxicity of some tubulysins also has the
5 disadvantage that a high general toxicity as well as a low selectivity against
normal cells is observed. In an attempt to lower the toxicity of the
tubulysins and to enhance their selectivity, polymer conjugates and
bioconjugates of tubulysins were developed (see e.g. WO2004/005326) which
10 exhibit a higher selectivity by a given cytotoxicity as well as lower toxicity
as compared to the unconjugated compounds. Herewith, predominantly
cancer cells are targeted in the human and the animal body and healthy
tissue is not affected.

Thus, the therapeutic potential of tubulysin derivatives and
(antibody) conjugates is very promising. Unfortunately, tubulysin
15 derivatives are very difficult to obtain by fermentation because of poor
yields and cumbersome isolation procedures. Whereas different approaches
are available for their complete chemical synthesis, these are quite lengthy
and provide a low overall yield.

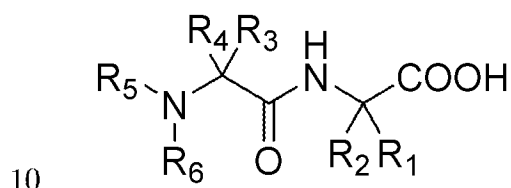
20 The present inventors therefore set out to develop a new synthetic approach
towards potent novel tubulysin derivatives. In particular, they aimed for a
synthetic procedure which is short (in numbers of steps), cheap, allows for
full stereoisomer control and has a high yield, preferably at a gram scale.

25 At least some of these goals could be met by the provision of a highly
efficient synthesis route (overall yield nearly 28%) based on only four
building blocks, three of which (compounds A, B and C) are reacted in a
multicomponent Passerini reaction. The tubulysin derivatives thus obtained
are super potent and can be attached towards biological matter (e.g. mAbs)
30 through at least 3 different linker positions. Furthermore, the synthetic

method offers full stereo-control. The new approach allows to fine-tune the properties of tubulysin ADCs in a much better way than offered by current products.

Accordingly, the invention provides method for preparing a tubulysin
5 derivative, comprising reacting compounds A, B and C in a 3-component
Passerini reaction,

wherein compound A is a carboxylic acid according to the general
formula A



wherein

R₁ represents a substituted or unsubstituted alkyl; a
substituted or unsubstituted cycloalkyl; or a substituted or
15 unsubstituted benzyl,

R₂ represents H, a substituted or unsubstituted alkyl, or
a substituted or unsubstituted cycloalkyl;

R₃ represents a substituted or unsubstituted alkyl; a
substituted or unsubstituted cycloalkyl; or a substituted or
20 unsubstituted benzyl;

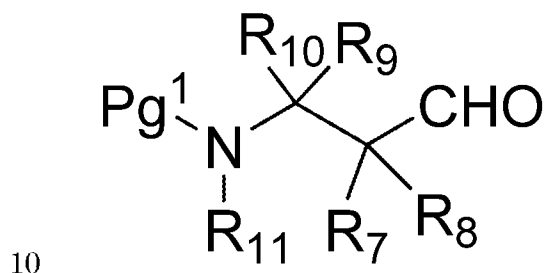
R₄ represents H, a substituted or unsubstituted alkyl, a
substituted or unsubstituted cycloalkyl, a substituted or
unsubstituted benzyl;

R₅ represents a substituted or unsubstituted alkyl or a
25 substituted or unsubstituted cycloalkyl; preferably a substituted
or unsubstituted cycloalkyl;

or wherein R_4 and R_5 are connected to form a 4 to 7 membered ring;

R_6 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; a substituted or unsubstituted benzyl, or COOR' , where R' is an optionally substituted alkyl, cycloalkyl, benzyl or aroyl moiety;

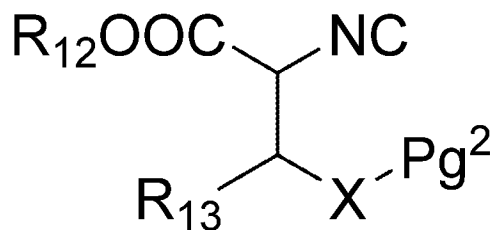
wherein compound B is an aldehyde according to the general formula B



wherein R_7 , R_8 , R_9 , R_{10} and R_{11} each independently represent H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl or a substituted or unsubstituted benzyl;

Pg^1 is an amine protecting group, preferably a carbamate, a substituted or an unsubstituted benzyl, or a substituted or an unsubstituted sulfonamide;

wherein compound C is an isocyanide according to the general formula C



wherein

R₁₂ represents a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

R₁₃ represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

X represents O, S, Se, or -NH-, preferably S; and

Pg² represents an X- protecting group, preferably selected from trityl, tert-butyl, adamantyl and substituted benzyl, more preferably trityl or tert-butyl.

Where it is indicated that a moiety may be substituted, such as by use of "unsubstituted or substituted" or "optionally substituted" phrasing as in "unsubstituted or substituted alkyl" or "optionally substituted benzyl," such moiety may have one or more independently selected substituents, preferably one to five in number, more preferably one or two in number. Substituents and substitution patterns can be selected by one of ordinary skill in the art, having regard for the moiety to which the substituent is attached, to provide compounds that are chemically stable and that can be synthesized by techniques known in the art as well as the methods set forth herein.

The term "substituents" refers to groups in which one or more hydrogen atoms are replaced with one or more moieties selected from the group consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate and phosphonate. One or more of the hydrogen atoms attached to carbon atom may be replaced by one or more halogen atoms, e.g. fluorine or chlorine or both, such as trifluoromethyl, difluoromethyl, fluorochloromethyl. In one embodiment, one or more hydrogen atoms is replaced by fluorine, chlorine, bromine or iodine atoms or OH, = O, SH, = S, NH₂, = NH or NO₂ groups. This expression further refers to groups which are exclusively or

additionally replaced with unsubstituted C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₁-C₆ heteroalkyl, C₃-C₁₀ cycloalkyl, C₂-C₉ heterocycloalkyl, C₆-C₁₀ aryl, C₁-C₉ heteroaryl, C₇-C₁₂ aralkyl or C₂-C₁₁ heteroaralkyl groups.

- 5 In a specific aspect, the term “substituted alkyl”, “substituted cycloalkyl” or “substituted benzyl” refers to an alkyl, cycloalkyl or benzyl that is substituted with 1-5 substituents, preferably 1 or 2 substituents, selected from the group consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, and phosphonate. In another specific aspect, one or more e.g. 1-5, 1-3 or 1, of the hydrogen atoms attached to carbon atom of the alkyl, cycloalkyl or benzyl is replaced by one or more halogen atoms, e.g. fluorine or chlorine or both, such as trifluoromethyl, difluoromethyl, fluorochloromethyl. Also encompassed are substituted alkyl, cycloalkyl and benzyl moieties wherein one or more e.g. 1-5, 1-3 or 1, hydrogen atom(s) is replaced by fluorine, chlorine, bromine or iodine atoms or OH, = O, SH, = S, NH₂, = NH or NO₂ groups.

The term “alkyl,” as used herein, unless otherwise specified, refers to a saturated straight or branched hydrocarbon chain of typically C₁ to C₁₀, and specifically includes methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, cyclohexylmethyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl, and the like.

In one embodiment, the alkyl is a lower alkyl. The term “lower alkyl,” as used herein, and unless otherwise specified, refers to a C₁ to C₄ saturated straight or branched alkyl group, including both substituted and unsubstituted forms as defined above. Unless otherwise specifically stated in this application, when alkyl is a suitable moiety, lower alkyl is preferred. Similarly, when alkyl or lower alkyl is a suitable moiety, unsubstituted alkyl or lower alkyl is preferred.

Alkyl groups can be optionally substituted with one or more moieties selected from the group consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate. One or more of the hydrogen atoms
5 attached to carbon atom on alkyl may be replaced by one or more halogen atoms, e.g. fluorine or chlorine or both, such as trifluoromethyl, difluoromethyl, fluorochloromethyl.

The term "cycloalkyl", as used herein, refers to a saturated or partially unsaturated (e.g. cycloalkenyl) cyclic group containing one or more rings
10 (preferably 1 or 2), the total of 3 to 14 ring carbon atoms, preferably 3 to 10 (especially 3 containing 4, 5, 6 or 7) ring carbon atoms. In one embodiment, it refers to a saturated hydrocarbon ring having 3-8 carbon atoms, preferably, 3-6 carbon atoms, such as cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl. The cycloalkyl group may also be substituted on the ring by
15 and alkyl group, such as cyclopropylmethyl and the like.

In one embodiment, the term "substituted benzyl" refers to a benzyl radical which is substituted by fluorine, chlorine, bromine, nitro, C₁-C₄ alkyl, C₁-C₄-halogenoalkyl or C₁-C₄ alkoxy. For example, the benzyl is substituted with chlorine, bromine, nitro, methyl or methoxy.

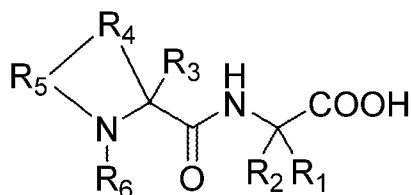
20 Unless particular stereoisomers are specifically indicated (e.g., by a bolded or dashed bond at a relevant stereocenter in a structural formula, by depiction of a double bond as having E or Z configuration in a structural formula, or by use stereochemistry-designating nomenclature), all
25 stereoisomers are included within the scope of the invention, as pure compounds as well as mixtures thereof. Unless otherwise indicated, individual enantiomers, diastereomers, geometrical isomers, and combinations and mixtures thereof are all encompassed.

As will be understood, any combination of preferred or specifically disclosed compounds A, B and C may be used in a method of the invention.

In one embodiment, the invention uses a carboxylic acid compound A of the
 5 general formula A, wherein R_1 represents isopropyl, tert-butyl, iso-butyl, sec-butyl, cyclopropylmethyl or cyclobutylmethyl; R_2 is H; R_3 is $-(CH_2)_n-$ CH_3 , wherein n is 3, 4 or 5; R_4 and R_5 are connected to form a 4 to 7
 membered ring; or R_5 is a substituted or unsubstituted cycloalkyl; and/or R_6
 is selected from the group consisting of benzyloxycarbonyl, 4-
 10 azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate and ethyl carbamate.

15 In a specific aspect, compound A is of the formula A' wherein R_4 and R_5 are connected, and together form a cyclic structure comprising 3, 4, 5, 6 or 7 carbon atoms.

20 Formula A'

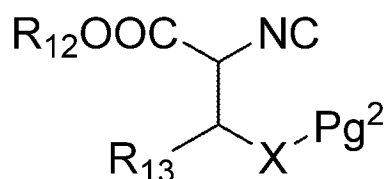


25 In one embodiment, R_3 represents H or methyl, and /or R_6 represents H, methyl, ethyl, propyl or cyclopropyl.

In a preferred embodiment, the method uses an aldehyde compound B according to the general formula B wherein R_7 is H; R_8 is H; R_9 is selected from the group consisting of isopropyl, cyclopropyl, cyclobutyl, isobutyl, sec-

butyl, tert-butyl and cyclopropylmethyl; R₁₀ is H; and/or R₁₁ is selected from the group consisting of methyl, ethyl, propyl, butyl, isopropyl, cyclopropyl and cyclopropylmethyl.

- 5 The Pg¹ moiety in compound B is an amine protecting group, preferably a carbamate, a substituted or an unsubstituted benzyl. For example, Pg¹ is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-
10 dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl.
- 15 Preferred isocyanide compounds C according to the general formula C include those wherein R₁₂ is methyl, ethyl or tert-butyl; R₁₃ is H or methyl, and/or wherein X represents S. The X-protecting group Pg² is preferably selected from trityl, tert-butyl, adamantyl and substituted benzyl, more preferably Pg² is trityl or tert-butyl.
- 20 An isocyanide compound according to the general formula C wherein X is S has been disclosed in Vishwanatha et al. (J. Org. Chem. 2017, 82, 9585-9594). The invention provides an isocyanide compound (suitably derived from cysteine) according to the general formula C
- 25



wherein the substituents are as defined herein above, and the use of the compound in the manufacture of a tubulysin derivate, preferably involving a 3-component Passerini reaction as herein disclosed.

Any one of the starting compounds for use in a method of the invention can be readily synthesized using conventional organic synthesis routes.

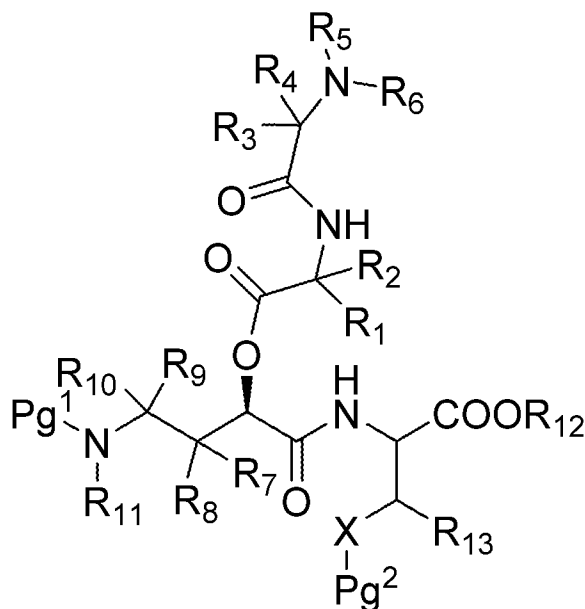
Exemplary synthetic routes for exemplary compounds A, B and C are provided in the Examples.

5

In a method of the invention, compounds A, B and C can be reacted in any suitable solvent or solvent mixture. In one embodiment, a non-coordinating solvent or solvent mixture is used. For example, good results can be obtained with CH₂Cl₂, CHCl₃, CCl₄, benzene, THF, CH₃CN, 1,4-dioxane, or
10 1,2-dichloroethane. In a specific aspect, compounds A, B and C are reacted in a mixture of CH₂Cl₂:THF (1:1 v/v). The reaction is most easily performed at room temperature. However, reaction conditions above or below room temperature are also encompassed. The reaction time can range from a few hours up to a few days. Typically, it ranges from about 16 to 60 h, like 24 to
15 48 h. The three starting compounds may be present in the starting reaction mixture about equimolar amounts, for example wherein A : B : C is 0.8-1.2: 0.8-1.2: 0.8-1.2, preferably 0.9-1.1:0.9-1.1:0.9-1.1. The Passerini reaction is preferably carried out from 1 mmol to 100 mmol scale.

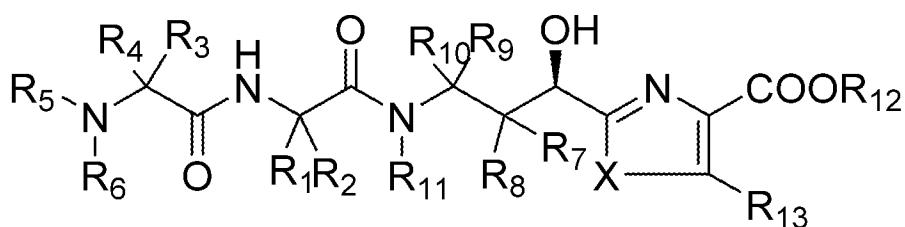
20 After completion of the Passerini reaction, the solvent(s) may be evaporated under reduced pressure. The reaction products are suitably purified through flash chromatography (e.g. cyclohexane/EtOAc 20:80 v/v). The by-product can also be recovered and coupled with dipeptide acid to form an ester.

25 Hence, in one embodiment a method of the invention further comprising isolating the 3-component Passerini reaction product of Formula D



wherein each of the (preferred) substituents is as defined herein above.

In order to obtain a tubulysin derivative, a method of the invention
 5 advantageously further comprises subjecting the Passerini reaction product
 of formula D to (a) an acyl migration reaction and (b) a cyclodehydration
 reaction of the Cys-amide. This will yield a thiazole compound of the general
 formula E having a hydroxyl moiety



10 .

wherein each of the (preferred) substituents is as defined herein above.

The acyl migration reaction is suitably performed in a two-step process
 15 involving exposure to diethylamine (DEA) followed by exposure to
 trimethylamine (TEA) using slight modification of existing methods. See for

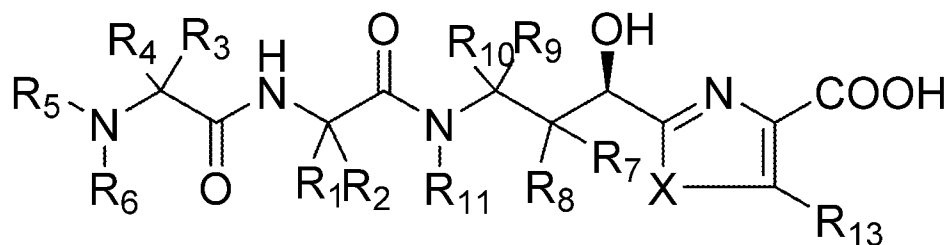
example Fauré et al.(Org. Lett. 2009, 11(5), pp 1167-1170) disclosing conditions for Amine Deprotection–Acyl Migration.

Cyclodehydration of the Cys-amide may also be performed in a two-step
 5 process, preferably involving incubation in the presence of TiCl_4 to obtain a thiazoline-containing compound, followed by oxidation in the presence of MnO_2 to obtain a thiazole moiety. Activated MnO_2 having a pore size of ≤ 5 microns is preferred.

10 The order in which the acyl migration reaction and the cyclodehydration reaction are performed are not critical. In one embodiment, acyl migration precedes cyclodehydration. In another embodiment, cyclodehydration precedes acyl migration. Preferably, the method comprises cyclodehydration, then oxidation followed by Fmoc deprotection and acyl
 15 migration.

As a next step, a method according to the invention advantageously further comprises hydrolyzing the R_{12} -containing ester of the general formula E to obtain a carboxylic acid compound of the general formula F

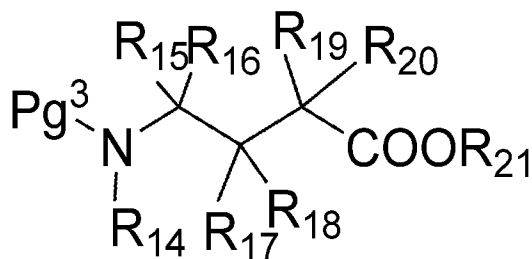
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wherein each of the (preferred) substituents is as defined herein above.

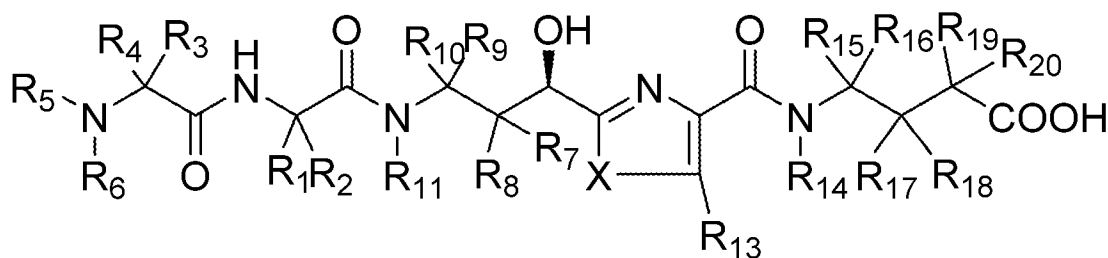
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Still further, the carboxylic acid compound of the formula F may be reacted with a compound of the general formula G



followed by removal of protecting moiety R₂₁, to obtain a compound of the general formula H

5



wherein R₁₄, R₁₅, R₁₆, R₂₀ and protecting moiety R₂₁ each independently represent H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl, R₁₇ and R₁₈ each independently represent H, F, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl and R₁₉ represents H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl.

15

Pg³ of compound G is an amine protecting group, preferably a carbamate, a substituted or unsubstituted benzyl, preferably selected from the group consisting of tertbutyl sulfine, benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl

20

carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl.

In a preferred embodiment, R₁₄ represents H;

5 R₁₅ represents H, benzyl, (substituted) benzyl;

R₁₆ represents H, benzyl, substituted benzyl (if R₁₅ is H) and/or

R₁₇ and R₁₈ represent H or F.

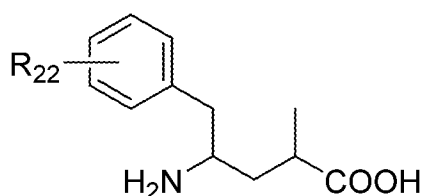
R₁₉ and/or R₂₀ are preferably independently selected from the group consisting of methyl, ethyl, propyl, butyl, isopropyl, cyclopropyl and

10 cyclopropylmethyl.

R₂₁ is preferably H, methyl or ethyl.

In a specific embodiment, the carboxylic acid compound of the formula F is reacted with tubuphenylalanine of the formula

15



or a salt thereof, such as the Na, K, Li or Ca salt,

wherein R₂₂ is H, OH, F or NO₂, preferably H, OH or F.

20 A further aspect of the invention relates to a method for providing a tubuphenylalanine of the above formula using (S)-(-)-methylsuccinic anhydride as intermediary compound. This method typically only comprising 6 steps, is shorter than the current synthetic procedures used and moreover has a better yield (up to nearly 60%) and increased

25 stereoselectivity.

In one embodiment, the method comprises the steps of :

(i) refluxing (S)-(-)-methylsuccinic acid in the presence of acetyl chloride to obtain (S)-(-)-methylsuccinic anhydride

(ii) opening of the succinic anhydride in the presence of.
(substituted) benzyl magnesium chloride and Cu I to obtain a keto-
5 pentanoic acid

(iii) dissolving the keto-pentanoic acid in an alcoholic solvent and refluxing in the presence of a strong acid to yield the corresponding keto-pentanoic ester as oil;

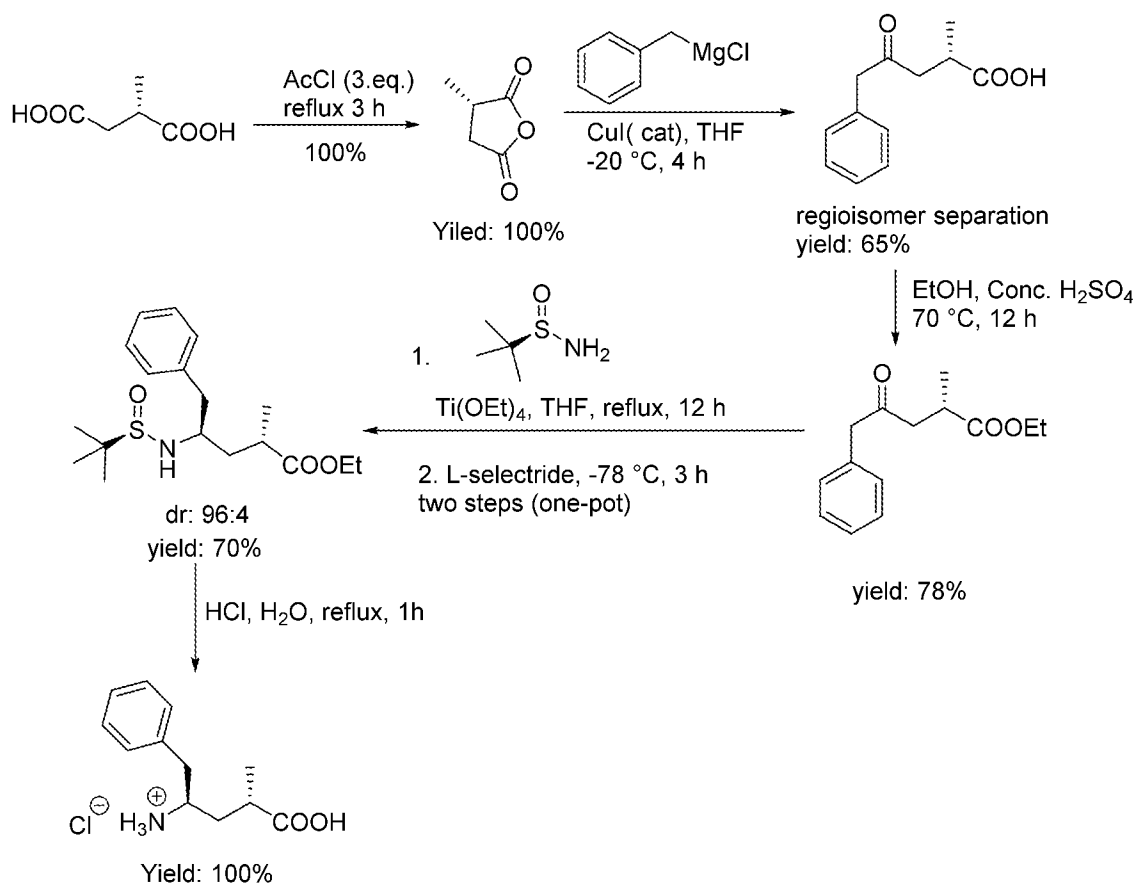
(iv) reacting the keto-pentanoic ester with ((S)-*tert*-
10 butylsulfinyl)amine in the presence of $\text{Ti}(\text{OEt})_4$

(v) cooling the reaction mixture of (iv) to a temperature below about -75°C ; and

(vi) adding L-selectride in a dropwise fashion to obtain (2*S*, 4*R*)-4-
(((S)-*tert*-butylsulfinyl)amino)-2- methyl-5-(substituted)phenylpentanoate.
15

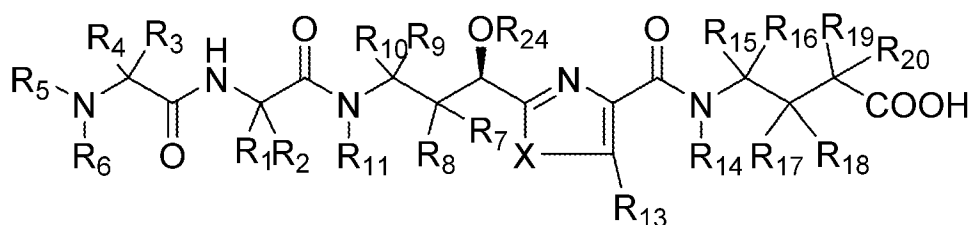
An exemplary method for providing a tubuphenylalanine compound is depicted in the following scheme:

17



A method of the invention for providing a tubulysin derivative may further comprise the acylation of the hydroxyl group a compound of the general formula H to obtain an ac(et)ylated compound of the general Formula I

5



wherein R_{24} represent acetyl, acyl (substituted) alkyl, acyl cycloalkyl, or acyl benzyl, preferably acetyl or acyl derivative of methyl, ethyl, tert-butyl or benzyl.

10

A method of the present invention allows for the introduction of a variety of different linker types used in the conjugation of payloads to small molecules, polymers, peptides, proteins, antibodies, antibody fragments etc. can be adopted and thereby, many different conjugation methods can be applied.

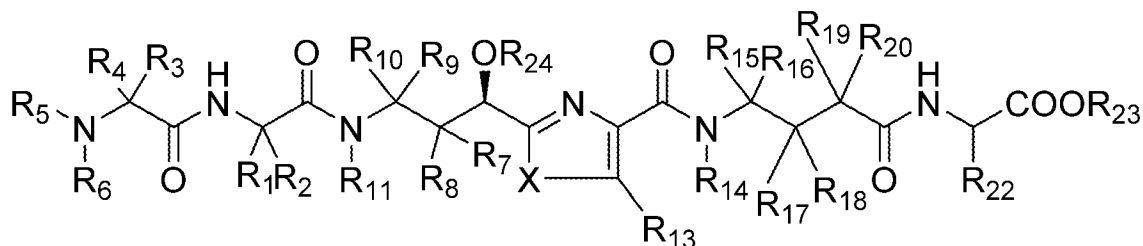
5 Spacer systems at different positions can be used either directly for conjugation by using different conjugation technologies such as chemical conjugation methods known in the art, or enzymatic conjugations using transglutaminases, sortases or other enzymes or which can be used in combination with commonly described linker systems known in the art .

10 More in particular, a method as herein disclosed provides tubulysin derivatives that can be attached toward biological matter, e.g. monoclonal antibodies, through at least 3 different linker positions. Herewith, the properties of, for example, ADC's can be fine-tuned in a better way as compared to existing products.

15 Therefore, the present invention particularly finds it use in the manufacture of a cytotoxic tubulysin derivative or tubulysin prodrug.

In one embodiment, a method as herein disclosed provides a conjugate comprising a tubulysin compound covalently linked to a targeting moiety
20 that specifically or preferentially binds to a chemical entity on a target cell, which target cell preferably is a cancer cell. Preferably, the targeting moiety is an antibody—more preferably a monoclonal antibody; even more preferably a human monoclonal antibody—and the chemical entity is a tumor associated antigen. The tumor associated antigen can be one that is
25 displayed on the surface of a cancer cell or one that is secreted by a cancer cell into the surrounding extracellular space.

In one aspect, a method of the invention comprising a step of reacting the ac(et)ylated compound of the general Formula I with Glu-(OMe)₂ or
30 alternative amino acid ester to obtain a compound of the formula J

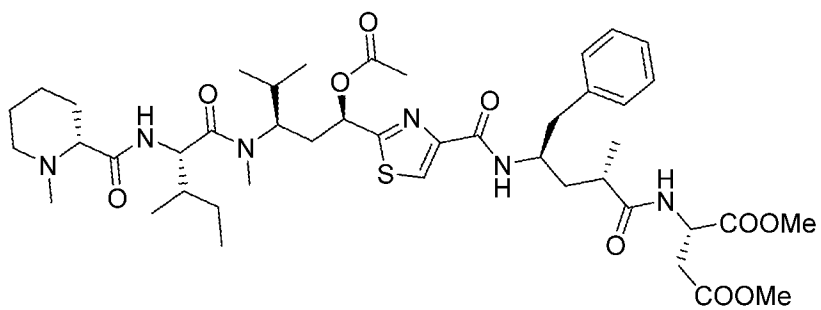
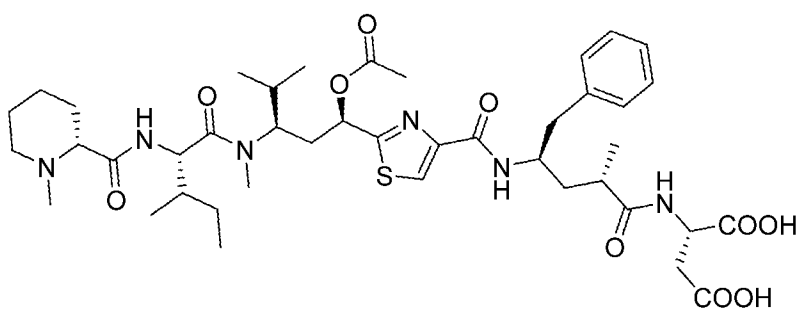
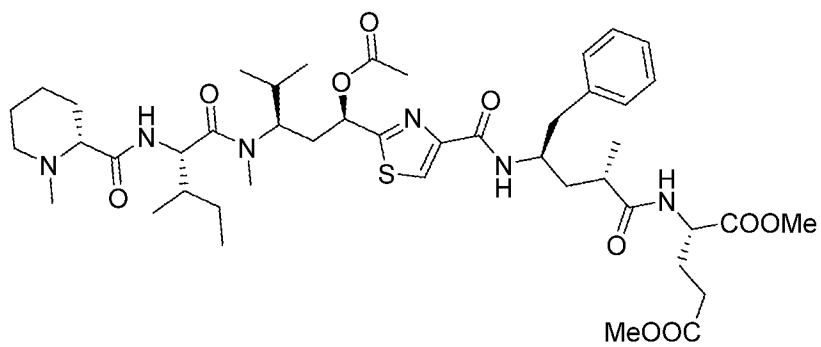
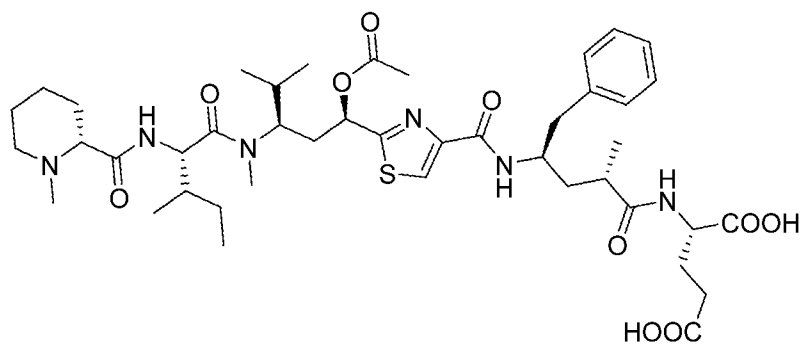


wherein R_{22} is H or an amino acid side chain, preferably $\text{CH}_2\text{-CH}_2\text{-COOZ}$

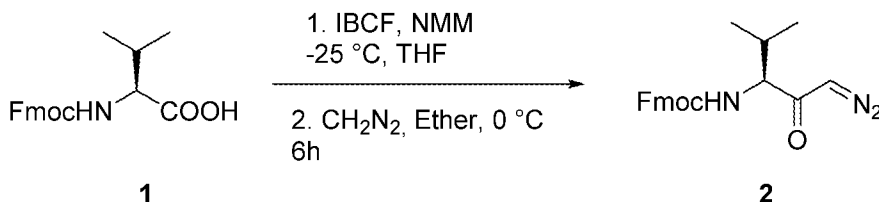
5 wherein Z is H or methyl,

R_{23} = H or a carboxyl protecting group, in particular methyl, ethyl, or tert-butyl; preferably H, methyl, ethyl or tert-butyl.

In a specific aspect, the method comprises the synthesis of a glutamic acid
 10 conjugate of tubulysin, which finds its use in a prodrug approach using
 glucarpidase or an enzyme showing a similar activity. For example, the
 method comprises the formation of a tubulysin-prodrug comprising one or
 more glutamate residue(s) linked to an amidic, urethanic or ureidic bond.
 For example, the invention provides one of the following exemplary
 15 compounds



EXPERIMENTAL SECTION

Example 1: Preparation of N^α-Fmoc-valine diazoketone

Fmoc-Val-OH (1.63 g, 4.79mmol) was dissolved in anhydrous THF (10mL) under nitrogen at 25 °C. N-Methylmorpholine (0.55mL, 5mmol) and isobutylchloroformate were then added to the mixture and after 5min of stirring, the mixture was cooled down to 78 °C. Anhydrous ether (10mL) was added and the fine suspension was filtered under N₂. Diazomethane (294mg, 7mmol) was added dropwise and the reaction was allowed to stir for 2 h at room temperature. After evaporation of the solvent, the residue was dissolved in CH₂Cl₂, and washed with water (3 X 10mL) before drying over MgSO₄ and concentration. Fmoc-Val-CHN₂ were purified via flash chromatography using EtOAc/hexane (30/70, v/v).

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 95%

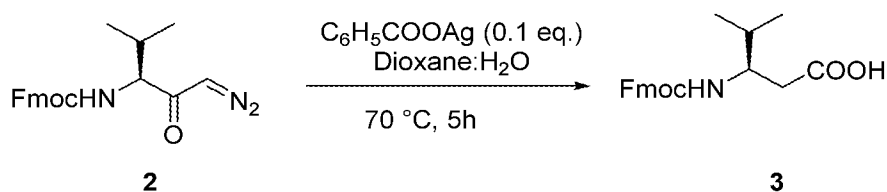
[α]_D²⁵ = +16.3 (c 1, CHCl₃)

MS (ESI) m/z calculated for C₂₁H₂₁N₃O₃ [M+Na]⁺ : 386.14, found 386.10

¹H NMR (500 MHz, CDCl₃): δ 8.43 -6.91 (8H, m), 6.24 (br, s, 1H), 5.07 (d, *J* = 6.9 Hz, 1H), 4.70 (d, *J* = 2.0 Hz, 2H), 4.48 – 4.43 (m, 1H), 2.54 (h, *J* = 6.8 Hz, 1H), 0.93 (dd, *J* = 24.9, 6.8 Hz, 6H).

¹³C NMR (100 MHz, CDCl₃): 191.2, 158.6, 144.2, 139.6, 126.9, 126.8, 123.8, 122.7, 67.0, 65.9, 60.6, 48.4, 31.2, 19.4.

Ref: Tetrahedron Letters 45 (2004) 8603–8606.

Example 2: Synthesis of Fmoc-β-Val-OH

A solution of **2** (1.0 mmol) in 1, 4-dioxane (10 mL) and water (5 mL) was
 5 treated with silver benzoate (0.02 mmol). It was refluxed at 70 °C for 5 h
 and then filtered. The solvent was evaporated under reduced pressure. The
 residue was dissolved in aqueous sodium carbonate (10%, 20 mL) and
 washed with diethyl ether (2 X 30 mL). The aqueous layer was acidified to
 pH 2–3 (using 2N HCl) and extracted with ethyl acetate (3 X 25 mL). The
 10 combined organic layer was washed with water and dried over anhydrous
 sodium sulphate and evaporated. The residue was recrystallized using ethyl
 acetate and *n*-hexane to give **3**. (95% yield).

TLC: 0.3 (CH₂Cl₂/ MeOH, 10:0.5),

Yield: 98%

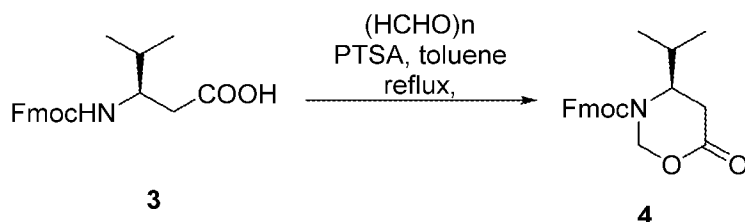
15 $[\alpha]_{\text{D}}^{25} = + 25.5$ (*c* 1, CHCl₃)

MS (ESI) *m/z* calculated for C₂₁H₂₃NO₄ [M+Na]⁺ : 376.15, found 376.08

¹H NMR (500 MHz, CDCl₃): δ 7.79- 7.43 (m, 8 H), 4.94 (s, 1H), 4.70 (d, *J* =
 5.6 Hz, 2H), 4.43 (q, *J* = 7.0 Hz, 1H), 4.32 – 4.25 (m, 1H), 2.79 (dd, *J* = 12.4,
 7.0 Hz, 1H), 2.67 – 2.53 (m, 2H), 0.81 (dd, *J* = 25.1, 6.8 Hz, 6H).

20 ¹³C NMR (100 MHz, CDCl₃): 174.4, 158.5, 144.2, 139.6, 126.9, 126.8, 123.8,
 122.7, 67.0, 58.9, 48.4, 34.6, 33.6, 19.1.

Ref: Syn. Commun., 2003, 33, 3089–3096.

Example 3: Synthesis of oxazolidinone 4

Fmoc- β -Val **3** (1.0 mmol) was suspended in 20 mL of toluene, and
 5 paraformaldehyde (1 g) and p-toluenesulfonic acid (100 mg) were added. The mixture was refluxed for 30 min with azeotropic water removal. The solution was cooled, washed with 1 N aqueous NaHCO₃ (2 X 25 mL) and dried over Na₂SO₄. Concentration in vacuo to yield **4** as yellow gum (yield: 88%).

10 TLC: 0.38 (Petroleum ether/EtOAc, 7:3)

Yield: 80%

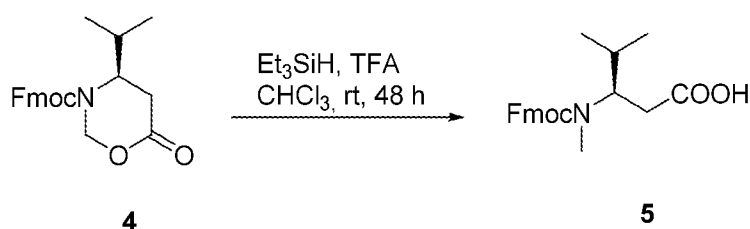
$[\alpha]_{\text{D}}^{25} = -15.9$ (*c* 1, CHCl₃)

MS (ESI) *m/z* calculated for C₂₁H₂₃NO₄ [M+Na]⁺: 388,15, found 388.10.

Major rotamer: ¹H NMR (500 MHz, CDCl₃): δ 8.10-7.44 (m, 8 H), 6.28 (d, *J* = 11.5 Hz, 1H), 6.20 (d, *J* = 11.5 Hz, 1H), 4.81 (q, *J* = 7.0 Hz, 1H), 4.70 (d, *J* = 5.7 Hz, 2H), 4.36 – 4.29 (m, 1H), 2.69 – 2.53 (m, 2H), 2.46 (dd, *J* = 14.6, 7.0 Hz, 1H), 0.83 (dd, *J* = 25.1, 6.8 Hz, 6H).

Major rotamer: ¹³C NMR (100 MHz, CDCl₃): δ 169.8, 158.3, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 73.2, 67.4, 52.2, 47.9, 38.2, 32.1, 19.1.

20 Ref: *J. Org. Chem.*, 1983, 48, 77-81.

Example 5: Synthesis of N-methyl-Fmoc- β -Val-OH 5

The oxazolidinone **4** (3.0 mmol) was dissolved in 15 mL of CHCl_3 , and 15 mL of trifluoroacetic acid and triethylsilane (1.43 mL, 1.04 g, 9.0 mmol) were added. The solution was stirred at room temperature for 48 h followed by concentration in vacuo to an oil. The oil was dissolved in CH_2Cl_2 and
 5 reconcentrated several times. The resultant oil was purified on silica gel flash column chromatography (PE/ EA 100:0 to 20:80).

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 85%

$[\alpha]_{\text{D}}^{25} = -32.5$ (c 1, CHCl_3)

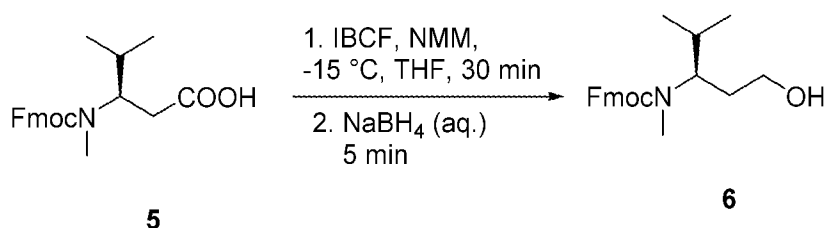
10 MS (ESI) m/z calculated for $\text{C}_{22}\text{H}_{25}\text{NO}_4$ $[\text{M}+\text{Na}]^+$: 390.16, 390.10.

Major rotamer: ^1H NMR (500 MHz, CDCl_3): δ 8.25-7.43 (m, 8H), 4.70 (d, J = 4.9 Hz, 2H), 4.16 (q, J = 7.0 Hz, 1H), 3.09 (s, 3H), 2.65 – 2.50 (m, 2H), 2.36 (dd, J = 12.4, 7.0 Hz, 1H), 0.88 (dd, J = 25.0, 6.8 Hz, 6H).

Major rotamer: ^{13}C NMR (100 MHz, CDCl_3): δ 174.0, 156.0, 144.8, 139.1,
 15 127.0, 126.8, 123.8, 122.7, 67.4, 53.6, 47.9, 34.2, 31.2, 19.1.

Ref: *J. Org. Chem.*, 1983, 48, 77-81.

Example 6: Synthesis of **6**



20

A solution of **5** (25 mmol) in THF (50 mL) was cooled to -15 °C (ice/ salt bath) under a nitrogen atmosphere. NMM (25 mmol, 2.78 mL, 1 equiv) and IBCF (25 mmol, 3.40 mL, 1 equiv) were added successively in a dropwise manner. After 30 h, the reaction mixture was filtered. The filtrate was
 25 cooled to -15 °C (ice/ salt bath), and a solution of NaBH_4 (37.5 mmol, 1.42 g, 1.5 equiv) in H_2O (12.5 mL) was added in one portion. After complete reduction (TLC analysis), the suspension was diluted with EtOAc and H_2O

(1:1). The organic layer was separated and the aqueous layer was extracted with EtOAc (2X 25 mL) and the combined organic layer was dried over Na₂SO₄ and evaporated under vacuum to yield **6** as gummy solid.

TLC: 0.25 (Petroleum ether/EtOAc, 5:5)

5 Yield: 96%

$[\alpha]_D^{25} = +7.6$ (*c* 1, CHCl₃)

MS (ESI) *m/z* calculated for C₂₂H₂₇NO₃ [M+Na]⁺: 376.18 (M + Na), found 376.08

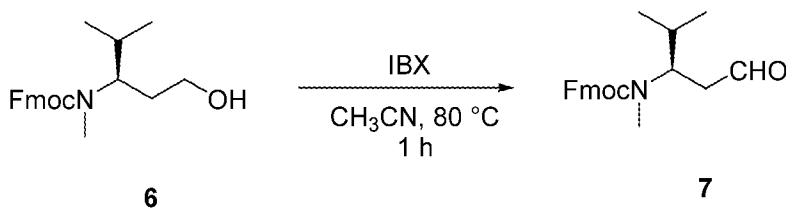
Major rotamer: ¹H NMR (500 MHz, CDCl₃): δ 8.10-7.15 (m, 8 H), 4.70 (d, *J* = 5.2 Hz, 2H), 3.84 – 3.73 (m, 1H), 3.69 – 3.57 (m, 2H), 3.11 (s, 3H), 2.67 – 2.53
10 (m, *J* = 6.8 Hz, 1H), 2.16 – 2.01 (m, 2H), 1.41 (t, *J* = 5.5 Hz, 1H), 0.90 (dd, *J* = 25.1, 6.8 Hz, 6H).

Major rotamer: ¹³C NMR (100 MHz, CDCl₃): δ 156.0, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 67.4, 59.5, 55.2, 47.9, 32.8, 31.2, 30.4, 19.1.

Ref: *J. Org. Chem.*, 2001, 66, 8454-8462

15

Example 7: Synthesis of Aldehyde (Exemplary compound B)



Alcohol **6** (1.00 mmol) was dissolved in CH₃CN (7 mL) and IBX (2.00 mmol)
20 was added. The resulting suspension was immersed in an oil bath set to 80 °C and stirred vigorously open to the atmosphere. After 2 h (TLC monitoring), the reaction was cooled to room temperature and filtered through a medium glass frit. The filter cake was washed with 3 × 2 mL of ethyl acetate, and the combined filtrates were concentrated and purified by
25 flash column chromatography to yield aldehyde **7** as a yellow gum.

TLC: 0.55 (Petroleum ether/EtOAc, 8:2)

Yield: 94%

$[\alpha]_{\text{D}}^{25} = +11.4$ (c 1, CHCl_3)

MS (ESI) m/z calculated for $\text{C}_{22}\text{H}_{25}\text{NO}_3$ $[\text{M}+\text{Na}]^+$: 374.17, found 374.10

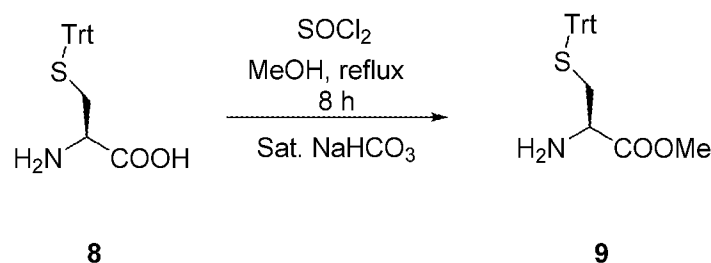
Major rotamer: ^1H NMR (500 MHz, CDCl_3): δ 9.76 (t, $J = 6.2$ Hz, 1H), 8.10-7.00 (m, 8H), 4.70 (d, $J = 5.3$ Hz, 2H), 4.33 – 4.27 (m, 1H), 3.92 (q, $J = 7.0$ Hz, 1H), 3.18 (s, 3H), 2.83 (ddd, $J = 12.3, 7.0, 6.1$ Hz, 1H), 2.67 – 2.53 (m, 2H), 0.89 (dd, $J = 25.0, 6.8$ Hz, 6H).

Major rotamer: ^{13}C NMR (100 MHz, CDCl_3): δ 199.5, 156.0, 144.8, 139.1, 127.0, 126.8, 123.8, 122.7, 67.4, 55.1, 47.9, 41.8, 31.2, 30.2, 19.1.

Ref: *Org. Lett.*, **2002**, 4, 3001-3003.

10

Example 8: Synthesis of methyl S-trityl-L-cysteinate, 9



To a solution of S-trityl-L-cysteine (1.0 g, 2.76 mmol) in 50 mL of methanol stirred at 0 °C was added thionyl chloride (1.50 mL, 0.206 mmol) in a drop wise fashion. The solution was allowed to warm up to room temperature and then refluxed at 80 °C for 5 h. The solvent was removed under reduced pressure and the crude product was extracted with ethyl acetate and washed with saturated sodium bicarbonate for several times. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated to give ester as pale yellow gum.

TLC: 0.32 (Petroleum ether/EtOAc, 5:5)

Yield: 80%

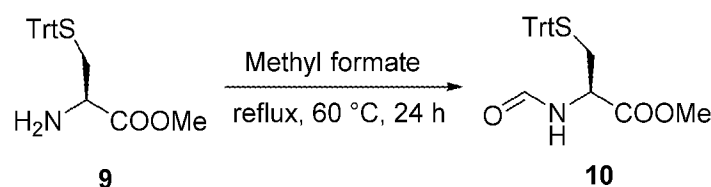
$[\alpha]_{\text{D}}^{25} = +20.4$ (c 1, CHCl_3)

MS (ESI) m/z calculated for $\text{C}_{23}\text{H}_{23}\text{NO}_2\text{S}$ $[\text{M}+\text{Na}]^+$: 400.13, found 400.10

^1H NMR (500 MHz, CDCl_3): δ 7.68 – 7.61 (m, 6H), 7.40 – 7.32 (m, 6H), 7.27 – 7.19 (m, 3H), 3.79 (t, $J = 7.0$ Hz, 1H), 3.71 (s, 3H), 3.64 (dd, $J = 12.3, 7.0$ Hz, 1H), 2.82 (dd, $J = 12.4, 7.0$ Hz, 1H), 2.19 (s, 2H).

^{13}C NMR (100 MHz, CDCl_3): δ 172.0, 144.9, 129.0, 128.8, 128.4, 127.5, 126.5, 126.2, 67.4, 53.5, 52.4, 33.5.

Example 9: Synthesis of methyl N-formyl-S-trityl-L-cysteinate, 10



Amine **9** (1.0 g, 2.65 mmol) was dissolved in methyl formate (10 mL, solvent) and the assembly was allowed to reflux at 60 °C until TLC showed complete consumption of starting material (usually 24 h). The solvent was evaporated and the product was purified through column chromatography to yield formyl ester **10** as a white solid.

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 95%

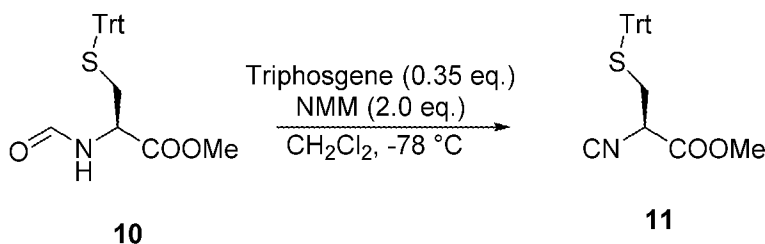
$[\alpha]_{\text{D}}^{25} = +9.6$ (c 1, CHCl_3)

MS (ESI) m/z calculated for $\text{C}_{24}\text{H}_{23}\text{NO}_3\text{S}$ $[\text{M}+\text{Na}]^+$: 428.12, found 428.10

^1H NMR (500 MHz, CDCl_3) δ 7.95 (d, $J = 1.3$ Hz, 1H), 7.50 – 7.11 (m, 16H), 6.14 (d, $J = 8.1$ Hz, 1H), 4.64 (dt, $J = 8.2, 5.2$ Hz, 1H), 3.68 (s, 3H), 2.77 (dd, $J = 12.7, 5.8$ Hz, 1H), 2.69 (dd, $J = 12.9, 6.5$ Hz, 1H).

^{13}C NMR (126 MHz, CDCl_3) δ 170.3, 160.4, 144.1, 129.4, 128.0, 128.0, 126.9, 126.8, 77.3, 77.1, 76.8, 67.0, 52.6, 49.7, 33.5.

Example 10: Synthesis of methyl (*R*)-2-isocyano-3-(tritylthio)propanoate (11) (Exemplary compound C)



5 A solution of *N*-formyl Cys(Trt)-methyl ester (1.0 eq) in CH₂Cl₂ (5.0 mL), was cooled to -78 °C. *N*-methylnmorpholine (2.0 eq) was added. After 5 min triphosgene (0.35 eq.) in CH₂Cl₂ (5.0 mL) was added drop wise and the reaction mixture was stirred for 3h at -78 °C (TLC analysis). Saturated NaHCO₃ solution (10 mL) was added at same temperature and allowed to

10 warm to room temperature. The reaction mixture was extracted with CH₂Cl₂, the organic extracts were separated, dried over anhydrous Na₂SO₄, filtered, and concentrated. The solution was diluted with diethyl ether (10 mL) and stored -15 °C for 5 h resulted in pure solid of isocyanide **11** which was collected by filtration.

15 TLC: 0.4 (Petroleum ether/EtOAc, 9:1)

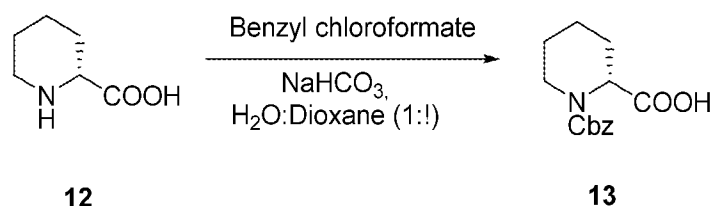
Yield: 84%

$[\alpha]_{\text{D}}^{25} = +55.2$ (*c* 1, CHCl₃)

MS (ESI) *m/z* calculated for C₂₄H₂₁NO₂S [M+Na]⁺: 410.11, found 410.25

¹H NMR (500 MHz, CDCl₃): ¹H NMR (500 MHz, CDCl₃) δ 7.57 – 7.06 (m, 16H), 3.70 (s, 3H), 3.34 (ddd, *J* = 7.7, 5.8, 1.6 Hz, 1H), 2.89 – 2.63 (m, 2H).

20 ¹³C NMR (126 MHz, CDCl₃) δ 165.6, 160.9, 143.9, 129.4, 129.2, 128.2, 128.0, 128.0, 127.9, 127.1, 67.5, 55.3, 53.4, 34.2.

Example 11: Synthesis of Acid component**11.1 Synthesis of Cbz-(D)-Pic-OH**

5 D-picolinic acid (30 mmol) and NaHCO₃ (60.0 mmol) were dissolved in a solution of H₂O and dioxane (1:1, 60 mL) was cooled to 0 °C. Benzylchloroformate (33.0 mmol) was added dropwise and the resulting suspension was stirred overnight at room temperature. The reaction mixture was quenched with 1N HCl till pH 2. This solution was extracted
 10 with EtOAc (3 X 15 mL) treated with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure followed by recrystallization with diethyl ether afforded a white solid **13**.

TLC: 0.41 (Petroleum ether/EtOAc/TFA 5:5:0.1)

Yield: 95%

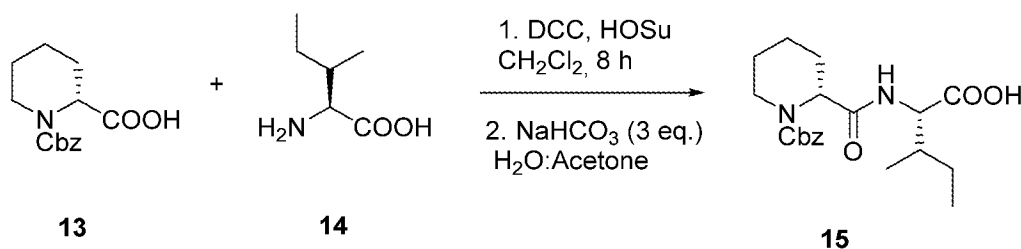
15 $[\alpha]_{\text{D}}^{25} = -155.2$ (*c* 1, CHCl₃)

MS (ESI) *m/z* calculated for C₁₄H₁₇NO₄ [M+Na]⁺: 286.10, found 286.18

Major Rotamer: ¹H NMR (500 MHz, CDCl₃): δ 7.38 – 7.27 (m, 5H), 5.83 (d, *J* = 12.4 Hz, 1H), 5.34 (d, *J* = 12.4 Hz, 1H), 5.05 (t, *J* = 6.9 Hz, 1H), 4.20 (dt, *J* = 12.5, 7.1 Hz, 1H), 3.32 (dt, *J* = 12.6, 7.1 Hz, 1H), 2.34 – 2.23 (m, 1H), 2.09
 20 – 1.93 (m, 2H), 1.74 – 1.58 (m, 2H), 1.58 – 1.49 (m, 1H).

Major Rotamer: ¹³C NMR (100 MHz, CDCl₃): δ 173.7, 157.2, 137.1, 128.5, 128.0, 66.7, 55.3, 43.3, 26.3, 24.5, 20.7.

11.2 Synthesis of Dipeptide Acid (exemplary Compound A)



- 5 A solution Cbz-picolinic acid (1.0 eq.) in anhydrous CH_2Cl_2 (10 mL) was cooled to 0 °C. N-hydroxysuccinimide (2.0 eq.) and DCC (1.2 eq.) were added. The reaction mixture was stirred at room temperature for 8h. The solution was filtered through pad of Cellite and the filtrate was evaporated under reduced pressure. In another flask Ile-OH (2 eq.) and NaHCO_3 (3.0 eq.) were
- 10 dissolved in 15 mL of water. To this solution the succinamide ester in 20 mL of DMF were added dropwise over 10 min. The reaction mixture was stirred at room temperature for 2 h and then diluted with 20 mL of water. The reaction mixture was washed with diethyl ether (2 X 10 mL). The aqueous phase was acidified to pH 2 using 1N HCl and extracted with EtOAc (3 X 20
- 15 mL). The combined organic layer was washed with brine, dried over MgSO_4 filtered and the solvent evaporated under reduced pressure to yield **15** (exemplary compound D).

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 79%

- 20 $[\alpha]_{\text{D}}^{25} = -65.6$ (*c* 1, CHCl_3)

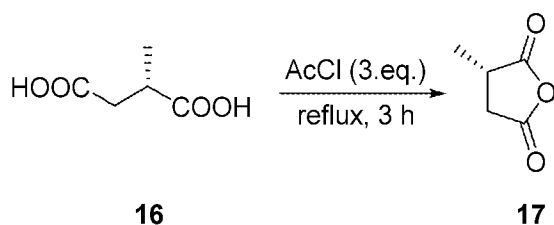
MS (ESI) *m/z* calculated for $\text{C}_{20}\text{H}_{28}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$: 376.11, found 376.10

- Major Rotamer: ^1H NMR (500 MHz, MeOD): δ 7.50 (s, 1H), 7.38 – 7.27 (m, 5H), 5.49 (d, $J = 12.4$ Hz, 1H), 5.37 (d, $J = 12.4$ Hz, 1H), 5.07 (t, $J = 7.0$ Hz, 1H), 4.43 – 4.33 (m, 2H), 3.53 (dt, $J = 12.6, 7.1$ Hz, 1H), 2.44 (dpd, $J = 12.3, 6.9, 2.7$ Hz, 1H), 2.15 – 1.96 (m, 2H), 1.82 – 1.55 (m, 3H), 1.53 – 1.28 (m, 3H), 1.01 (d, $J = 6.8$ Hz, 3H), 0.92 (t, $J = 8.0$ Hz, 3H).
- 25

Major Rotamer: ^{13}C NMR (100 MHz, MeOD): δ 175.2, 171.0, 157.2, 137.1, 128.5, 128.0, 66.7, 58.6, 55.6, 43.3, 36.1, 26.6, 24.5, 24.3, 20.8, 15.0, 11.6.

Example 12: Synthesis of (S)-(-)-methyl succinic anhydride

5



(S)-(-)-methyl succinic acid (10.0 mmol) and acetyl chloride (30.0 mmol) were placed in a single neck round bottomed flask and the assembly was refluxed on the steam bath for 3 h. The solution is allowed to cool undisturbed and is finally chilled in an ice bath. The succinic anhydride, which separates in beautiful crystals, is collected on a Büchner funnel, washed with two 75-cc. portions of cold ether, and dried in a vacuum desiccator.

TLC: 0.54 (Petroleum ether/EtOAc, 6:4)

Yield: 100%

15 $[\alpha]_{\text{D}}^{25} = -11.0$ (c 1, CHCl_3)

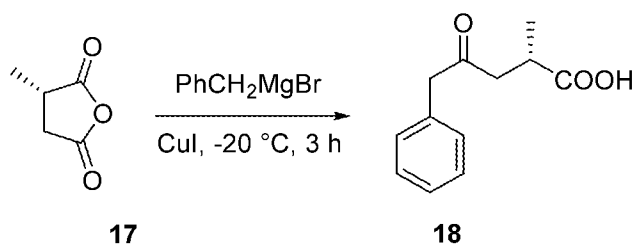
MS (ESI) m/z calculated for $\text{C}_5\text{H}_6\text{O}_3$ $[\text{M}+\text{Na}]^+$: 137.12, found 137.10

^1H NMR (500 MHz, CDCl_3): δ 3.48 (h, $J = 6.9$ Hz, 1H), 3.02 (dd, $J = 18.2, 7.0$ Hz, 1H), 2.56 (dd, $J = 18.2, 7.0$ Hz, 1H), 1.17 (d, $J = 6.8$ Hz, 3H).

^{13}C NMR (100 MHz, CDCl_3): δ 176.8, 172.9, 35.0, 30.3, 15.1.

20 Ref: *Org. Synth.* **1932**, 12, 66

Example 13: Synthesis of (S)-2-methyl-4-oxo-5-phenylpentanoic acid, 18



5 To a solution of anhydride **17** (10 mmol) and CuI (0.1 mmol) in dry THF (50 mL) to -20 °C. benzyl magnesium bromide (2.0 M in THF, 12.0 mmol) slowly to the reaction mixture. (A purple color forms, but it disappear after the addition is finished). The reaction mixture was stirred at same temperature for additional 3h and then room temperature for 1h. H₂O (50 mL) and 1N HCl (50 mL) were added and stirred for 5 mins. The reaction mixture diluted with was EtOAc (50 mL X 2) and filtered through pad of Cellite. The organic layer was separated, dried over MgSO₄ and evaporated under reduced pressure. The crude product was purified though column chromatography. The regioisomers were purified again to obtain desired product **18**.

TLC: 0.51 (Petroleum ether/EtOAc/TFA, 7:3:0.1)

Yield: 61%

$[\alpha]_{\text{D}}^{25} = -25.8$ (c 1, CHCl₃)

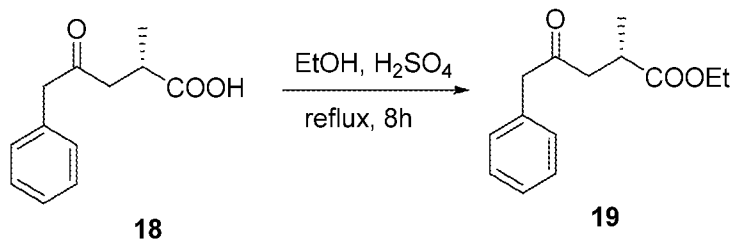
MS (ESI) m/z calculated for C₁₂H₁₄O₃ [M+Na]⁺: 229.08, found 229.12

20 ¹H NMR (500 MHz, CDCl₃): δ 7.38 – 7.28 (m, 4H), 7.28 – 7.20 (m, 1H), 3.71 (dt, *J* = 12.3, 1.0 Hz, 1H), 3.58 (d, *J* = 12.3 Hz, 1H), 2.98 (dd, *J* = 12.2, 7.0 Hz, 1H), 2.90 (dt, *J* = 13.6, 6.7 Hz, 1H), 2.61 (dd, *J* = 12.2, 6.8 Hz, 1H), 1.22 (d, *J* = 6.8 Hz, 3H).

¹³C NMR (100 MHz, CDCl₃): δ 209.5, 178.1, 136.7, 129.5, 129.0, 126.8, 48.7, 45.6, 36.0, 17.9.

Ref: *J. Med. Chem.*, **2007**, 50 (18), pp 4261–4264

Example 14: Synthesis of ethyl (S)-2-methyl-4-oxo-5-phenylpentanoate, 19



The ketoacid **18** (7.0 mmol) was dissolved in EtOH (40 mL), concentrated
 5 H₂SO₄ (1.1 mL, 21.0 mmol) was added at room temperature, and then the
 reaction mixture was refluxed overnight. After evaporation of the solvent,
 H₂O (10 mL) was added, and the mixture was neutralized with a 2 M
 aqueous NaOH solution and extracted with EtOAc (3 × 15 mL). The
 combined organic phases were dried (Na₂SO₄). After filtration and
 10 evaporation of the solvent, the expected ketoester obtained as oil.

TLC: 0.45 (Petroleum ether/EtOAc, 9.5:0.5)

Yield: 90%

$[\alpha]_{\text{D}}^{25} = -20.6$ (*c* 1, CHCl₃)

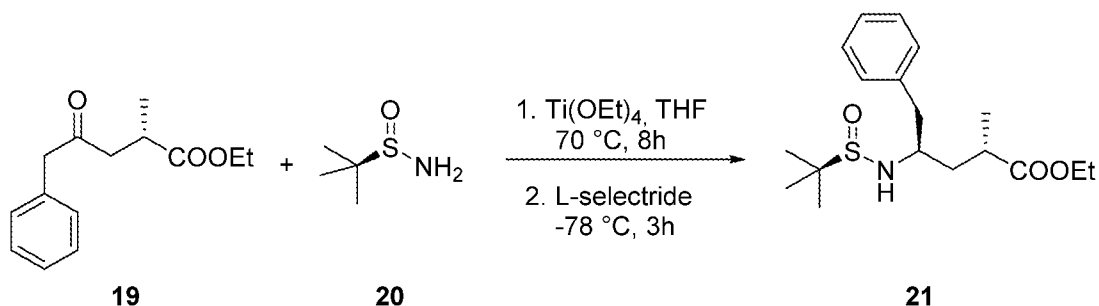
MS (ESI) *m/z* calculated for C₁₄H₁₈O₃ [M+Na]⁺: 257.11, found 257.10

15 ¹H NMR (500 MHz, CDCl₃): δ 7.38 – 7.27 (m, 4H), 7.27 – 7.20 (m, 1H), 4.68
 (dq, *J* = 12.1, 5.9 Hz, 1H), 3.83 (dt, *J* = 12.4, 1.0 Hz, 1H), 3.60 (dq, *J* = 12.1,
 6.0 Hz, 1H), 3.45 (d, *J* = 12.5 Hz, 1H), 3.35 (dd, *J* = 12.3, 7.0 Hz, 1H), 2.99
 (h, *J* = 6.9 Hz, 1H), 2.46 (dd, *J* = 12.4, 7.0 Hz, 1H), 1.26 – 1.18 (m, 6H).

¹³C NMR (100 MHz, CDCl₃): δ 209.5, 176.6, 136.7, 129.5, 129.0, 126.8, 61.5,
 20 48.7, 45.4, 36.0, 17.9, 14.1.

Ref: *J. Org. Chem.*, **2013**, 78, 3647

Example 15: Synthesis of ethyl (2S,4R)-4-(((S)-tert-butylsulfinyl)amino)-2-methyl-5-phenylpentanoate, **21**



5 Ketone (1.0 mmol) was added to a solution of **20** (1.5 mmol) and Ti(OEt)₄ (3.0 mmol) in THF (15 mL) at room temperature (rt). The reaction mixture was heated at 70 °C for 8h and the reaction conversion was followed by TLC. Once the reaction was determined to be complete by TLC, the mixture was cooled to room temperature and then to -78 °C. L-Selectride (1 M solution in

10 THF) was added dropwise. The reaction mixture was stirred at same temperature for 3 h. Once the reduction was determined to be complete by TLC, the reaction mixture was quenched by dropwise addition of MeOH at 0 °C until gas evolution was no longer observed. The crude reaction mixture was poured into an equal volume of brine while being rapidly stirred. The

15 resulting suspension was filtered through a plug of Cellite, and the filter cake was washed with EtOAc. The filtrate was washed with brine, and the brine layer was extracted with EtOAc (3×). The combined organic portions were dried (Na₂SO₄), filtered, and concentrated. The product **21** was purified by silica gel chromatography (hexanes/EtOAc).

20 TLC: 0.42 (Petroleum ether/EtOAc, 6:4)

Yield: 89%

$[\alpha]_D^{25} = +6$ (*c* 1, CHCl₃)

MS (ESI) *m/z* calculated for C₁₈H₂₉NO₃S [M+Na]⁺: 362.17, found 362.18

¹H NMR (500 MHz, CDCl₃): δ 7.32 – 7.24 (m, 2H), 7.24 – 7.13 (m, 3H), 4.78 (dq, *J* = 12.1, 6.0 Hz, 1H), 3.85 (s, 1H), 3.69 (dq, *J* = 12.2, 6.0 Hz, 1H), 3.60

25

(dtd, $J = 11.9, 7.0, 2.2$ Hz, 1H), 3.31 (ddt, $J = 12.5, 6.9, 1.1$ Hz, 1H), 2.92 (ddq, $J = 12.3, 7.0, 0.8$ Hz, 1H), 2.59 (h, $J = 6.9$ Hz, 1H), 2.28 (td, $J = 12.1, 6.9$ Hz, 1H), 2.13 – 2.05 (m, 1H), 1.29 – 1.20 (m, 15H).

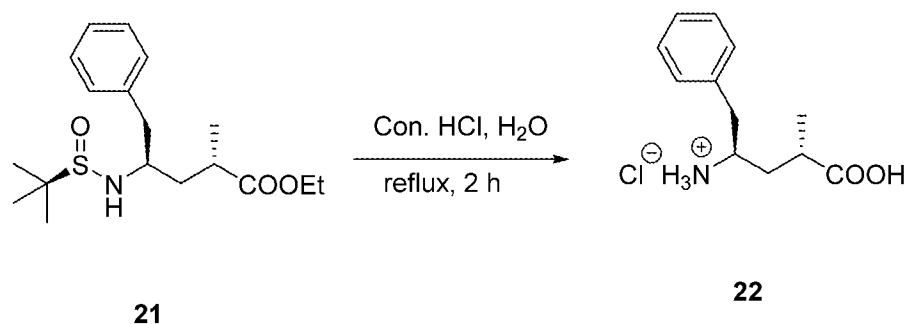
^{13}C NMR (100 MHz, CDCl_3): δ 177.6, 137.0, 129.3, 128.4, 126.3, 69.6, 61.5,

5 56.7, 39.9, 38.7, 37.7, 20.4, 18.1, 14.1.

Ref: *J. Org. Chem.*, **2007**, 72, 626-629

Example 16: Synthesis of Tubuphenylalanine (22) (Exemplary compound G).

10



H_2O (3.07 mL) was added to **21** (1.0 mmol), followed by conc. HCl (3.07 mL), and the reaction mixture was heated to 100 °C for 2h. The mixture was then allowed to cool to room temperature and concentrated under vacuum. The

15 resulting oil was triturated three times with Et_2O to give **22** (100%) as a white powder.

TLC: 0.31 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH}$, 9:1:0.2)

Yield: 95% $[\alpha]_{\text{D}}^{25} = +15.2$ ($c = 1$, MeOH)

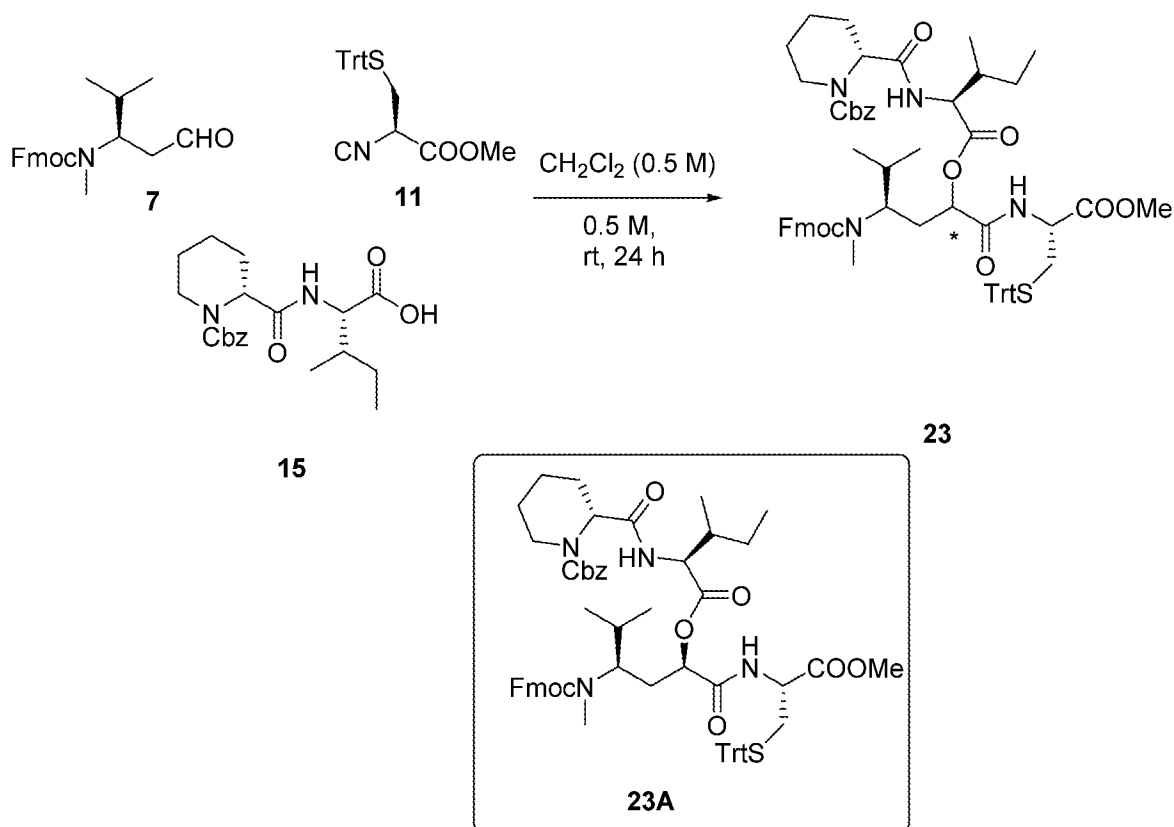
MS (ESI) m/z calculated for $\text{C}_{12}\text{H}_{17}\text{NO}_2$ $[\text{M}+\text{H}]^+$: 208.13, found 208.05

20 ^1H NMR (500 MHz, D_2O) δ 7.29 (m, 2H), 7.20 (t, 1H, $J = 7.3$ Hz), 7.22 (d, 2H, $J = 7.1$ Hz), 3.49 (m, 1H), 2.85 (dd, 1H, $J = 6.6, 14.2$ Hz), 2.82 (dd, 1H, $J = 7.6, 14.2$ Hz), 2.40-2.60 (m, 1H), 1.77-1.88 (m, 1H), 1.42-1.68 (m, 1H), 1.06 (d, 3H, $J = 7.0$ Hz).

^{13}C NMR (125 MHz, D_2O) δ 180.1, 135.2, 129.8, 128.8, 128.0, 51.6, 39.0,

25 36.5, 36.0, 17.0.

129.8, 130.1, 136.1, 180.7.

Example 17: Total Synthesis of 23

- 5 To a mixture of dipeptide acid **15** (1.0 eq), aldehyde **7** (1.0 eq) and isocyanide **11** (1.1 eq) in CH_2Cl_2 (2 mL) was stirred at room temperature for 24-48 h. The solvents were evaporated under reduced pressure and purified through flash chromatography (cyclohexane/EtOAc 20:80). The mixture of diastereomers **23** were purified again and pure diastereomer **23A** was used
- 10 further.

TLC: 0.34 (Petroleum ether/EtOAc, 7:3)

Yield: 80%

$[\alpha]_{\text{D}}^{25} = -96.5$ (*C* 1, CHCl_3)

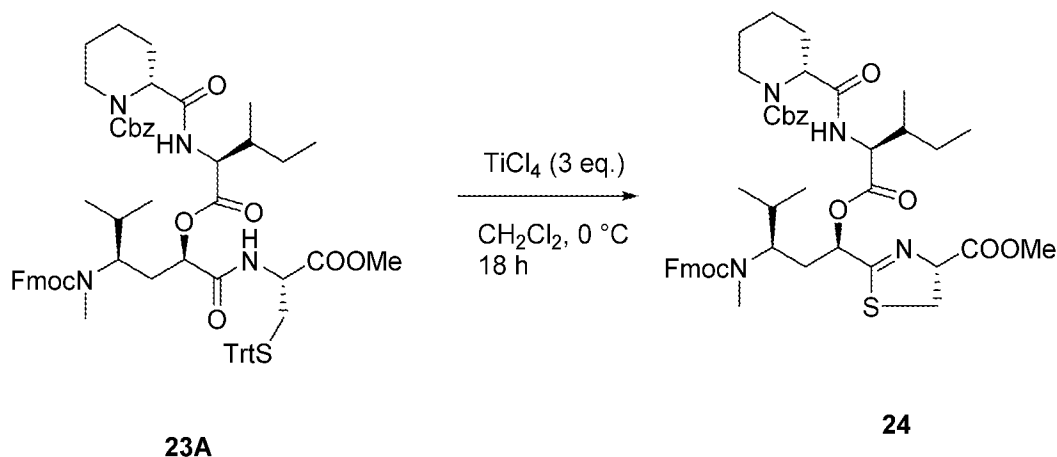
MS (ESI) *m/z* calculated for $\text{C}_{66}\text{H}_{74}\text{N}_4\text{O}_{10}\text{S}$ $[\text{M}+\text{Na}]^+$: 1137.2, found 1137.25

- 15 Major rotamer: ^1H NMR (500 MHz, CDCl_3): δ 7.93 – 7.84 (m, 3H), 7.82 (dt, $J = 7.2, 1.8$ Hz, 2H), 7.71 – 7.64 (m, 2H), 7.59 – 7.52 (m, 4H), 7.50 (s, 2H), 7.45 (tdd, $J = 7.5, 6.0, 1.5$ Hz, 2H), 7.42 – 7.13 (m, 15H), 5.01 (t, $J = 7.0$ Hz,

1H), 4.92 (d, $J = 7.0$ Hz, 1H), 4.82 – 4.72 (m, 2H), 4.72 – 4.67 (m, 2H), 4.49 (dt, $J = 12.5, 6.9$ Hz, 1H), 4.35 – 4.28 (m, 1H), 4.17 (ddd, $J = 11.9, 7.0, 2.5$ Hz, 1H), 3.71 (s, 3H), 3.51 – 3.41 (m, 1H), 3.33 (dd, $J = 12.4, 7.0$ Hz, 1H), 3.25 (s, 3H), 2.98 – 2.84 (m, 2H), 2.81 – 2.69 (m, 1H), 2.67 – 2.53 (m, $J = 6.8$ Hz, 1H), 2.07 – 1.91 (m, 2H), 1.91 – 1.44 (m, 6H), 1.02 – 0.85 (m, 12H).
Major rotamer: ^{13}C NMR (100 MHz, CDCl_3): δ 171.0, 169.9, 157.2, 156.0, 144.9, 139.1, 137.1, 128.8, 128.6, 128.5, 128.0, 127.5, 127.0, 126.8, 123.8, 122.7, 69.5, 66.7, 59.8, 55.6, 53.9, 53.5, 52.4, 47.9, 43.3, 35.5, 33.2, 32.2, 26.6, 24.5, 24.3, 20.8, 19.1, 15.0, 11.6.

10 *Ref. Org. Lett.*, **2009**, *11* (5), pp 1167–1170

Example 18: Synthesis of thiazoline, 24



A solution of S-trityl-protected cysteine N-amide **23A** (1.0 mmol) in dry
15 CH_2Cl_2 (10 mL) was treated with TiCl_4 (1.0 M solution in CH_2Cl_2 , 3.0 mmol) and stirred at 0 °C for 18 h. The reaction mixture was stirred vigorously with cold saturated aqueous NaHCO_3 (2 $\times$) for 30 min. The aqueous layer was extracted with CH_2Cl_2 , and the combined organic layers were dried over MgSO_4 , filtered, and concentrated. The resultant crude product was purified
20 by flash chromatography to afford **24** as a yellow foam.

TLC: 0.32 (Petroleum ether/EtOAc, 6:4)

Yield: 75%

$[\alpha]_{\text{D}}^{25} = -108.3$ (C 1, CHCl_3)

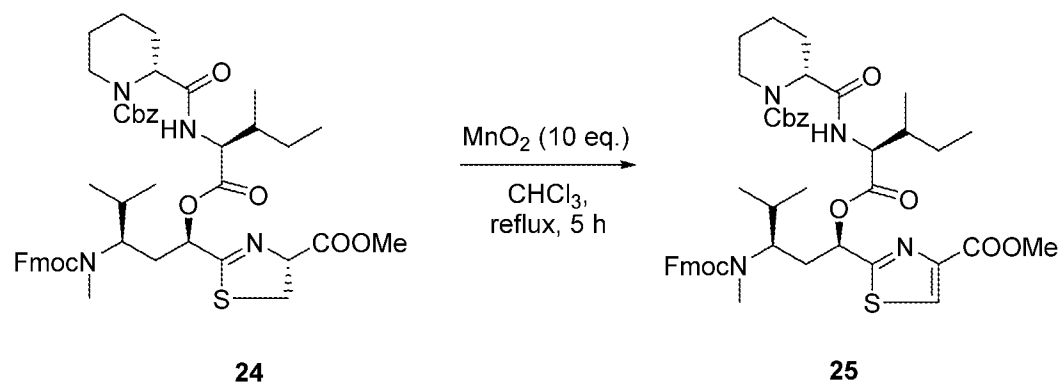
MS (ESI) m/z calculated for $C_{47}H_{58}N_4O_9S$ $[M+Na]^+$: 877.12, found 877.20

^1H NMR (500 MHz, $CDCl_3$): δ 7.85 – 7.76 (m, 2H), 7.59 – 7.48 (m, 4H), 7.44 (td, $J = 7.5, 1.5$ Hz, 1H), 7.40 – 7.27 (m, 6H), 5.06 (dt, $J = 26.4, 7.1$ Hz, 2H), 4.77 – 4.67 (m, 3H), 4.55 (t, $J = 6.9$ Hz, 1H), 4.42 (dt, $J = 12.5, 7.1$ Hz, 1H),
 5 4.27 (td, $J = 7.3, 0.8$ Hz, 1H), 4.08 (ddd, $J = 11.9, 7.0, 2.4$ Hz, 1H), 3.85 (dd, $J = 12.5, 7.0$ Hz, 1H), 3.71 (s, 3H), 3.40 (dd, $J = 12.5, 7.0$ Hz, 1H), 3.22 (s, 3H), 3.03 (dt, $J = 12.4, 7.1$ Hz, 1H), 2.81 – 2.69 (m, 1H), 2.67 – 2.53 (m, $J = 6.8$ Hz, 1H), 2.17 (ddd, $J = 12.4, 7.0, 2.4$ Hz, 1H), 2.02 – 1.90 (m, 2H), 1.83 (dp, $J = 13.0, 7.1$ Hz, 1H), 1.76 – 1.48 (m, 3H), 1.42 (dp, $J = 13.0, 7.1$ Hz, 1H), 1.23
 10 – 1.11 (m, 2H), 1.02 – 0.92 (m, 6H), 0.81 (d, $J = 6.8$ Hz, 3H), 0.72 (d, $J = 6.8$ Hz, 3H).

Major Rotamer: ^{13}C NMR (125 MHz, $CDCl_3$): 172.9, 172.1, 171.0, 170.1, 157.2, 156.0, 144.8, 139.1, 137.1, 128.5, 128.0, 127.0, 126.8, 123.8, 122.7, 74.0, 67.4, 66.7, 65.6, 59.8, 55.6, 53.5, 51.7, 47.9, 43.3, 35.8, 35.5, 34.3, 26.6,
 15 24.5, 24.3, 20.8, 19.1, 15.0, 11.6.

Ref: Org. Lett., 2000, 2, 3289.

Example 19: Synthesis of thiazole, 25



20

To a solution of **24** (1 mmol) in $CHCl_3$ (5 mL), activated MnO_2 (< 10 micron, 10 mmol) was added. The reaction mixture was refluxed at 70 °C for 5 h, then filtered through a short silica gel and celite column and washed with $CHCl_3$. The organic solution was concentrated. The resulting crude product

was purified by flash chromatography (EtOAc/hexanes = 1/3) to afford compound **25** as a white foam.

TLC: 0.41 (Petroleum ether/EtOAc, 7:3)

Yield: 99%

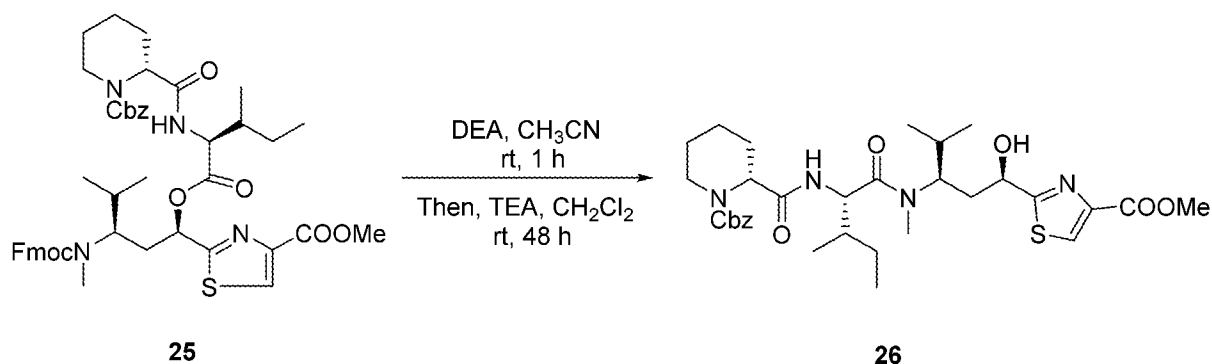
5 $[\alpha]_D^{25} = -33.2$ (C 1, CHCl_3)

MS (ESI) m/z calculated for $\text{C}_{47}\text{H}_{56}\text{N}_4\text{O}_9\text{S}$ $[\text{M}+\text{Na}]^+$: 875.21, found 875.20

^1H NMR (500 MHz, CDCl_3): δ 7.83 – 7.72 (m, 2H), 7.66 (dd, $J = 7.4, 1.5$ Hz, 1H), 7.55 – 7.40 (m, 4H), 7.37 – 7.28 (m, 5H), 7.16 (dd, $J = 7.5, 1.5$ Hz, 1H), 7.07 (td, $J = 7.5, 1.5$ Hz, 1H), 5.89 (t, $J = 7.0$ Hz, 1H), 4.79 (dd, $J = 6.9, 1.1$ Hz, 1H), 4.70 (d, $J = 7.0$ Hz, 2H), 4.44 (dt, $J = 12.6, 7.0$ Hz, 1H), 4.35 (t, $J = 7.2$ Hz, 1H), 4.26 (t, $J = 6.9$ Hz, 1H), 4.13 (ddd, $J = 11.2, 7.0, 4.2$ Hz, 1H), 3.96 (s, 3H), 3.16 (s, 3H), 2.81 – 2.71 (m, 2H), 2.67 – 2.45 (m, 3H), 2.02 – 1.80 (m, 4H), 1.68 – 1.55 (m, 1H), 1.55 – 1.39 (m, 3H), 1.09 (t, $J = 8.0$ Hz, 3H), 0.97 (d, $J = 6.8$ Hz, 3H), 0.72 (dd, $J = 24.0, 6.8$ Hz, 6H).

15 ^{13}C NMR (100 MHz, CDCl_3): δ 172.1, 171.0, 169.6, 162.4, 157.2, 156.0, 139.1, 137.1, 128.5, 128.0, 127.0, 126.8, 123.8, 122.7, 118.8, 67.4, 66.7, 63.8, 59.8, 55.6, 53.5, 52.0, 47.9, 43.3, 35.8, 35.5, 26.6, 24.5, 24.3, 20.8, 19.1, 15.0, 11.6.

Example 20: Fmoc deprotection and acyl migration: synthesis of 26



To a solution of **25** (1.0 mmol) in CH₃CN (12 mL) at 0 °C under Ar was added Et₂NH (8 mL). The solution was stirred at room temperature for 1 h. After complete deprotection of Fmoc group (TLC analysis) the mixture was evaporated and co-evaporated with CH₂Cl₂ to remove DEA. The crude crude product which was dissolved in CH₂Cl₂ (0.8 mL) and Et₃N (0.3 mL) was added and the resulting mixture was stirred at room temperature for 18 h. The crude hydroxyl derivative **26** was purified by column chromatography. The non-acylated amine was isolated and again stirred in presence of Et₃N for overnight.

TLC: 0.32 (Petroleum ether/EtOAc, 3:7)

15 Yield: 75%

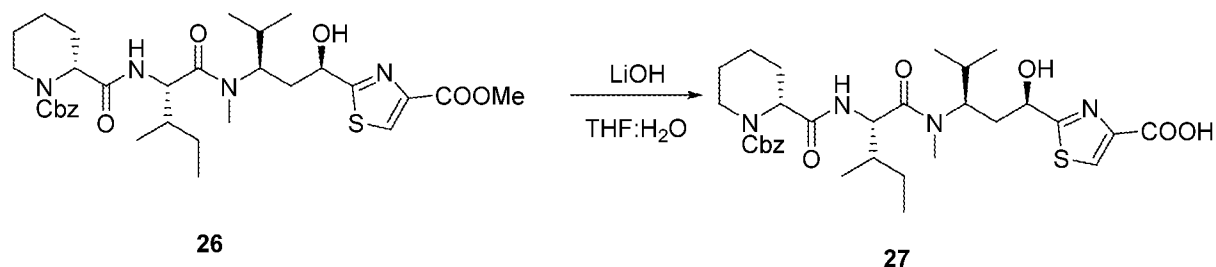
$$[\alpha]_{\text{D}}^{25} = +68.2 \text{ (C 1, MeOH)}$$

MS (ESI) m/z calculated for C₃₂H₄₆N₄O₇S [M+Na]⁺: 653.29, found 653.10

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.37 – 7.29 (m, 5H), 5.54 (d, J = 12.4 Hz, 1H), 5.38 – 5.31 (m, 1H), 5.12 (d, J = 6.7 Hz, 1H), 4.83 (td, J = 6.9, 4.6 Hz, 2H), 4.17 (dt, J = 12.4, 7.1 Hz, 1H), 3.96 (s, 3H), 3.82 (ddd, J = 11.7, 7.0, 2.7 Hz, 1H), 3.42 (dt, J = 12.6, 7.1 Hz, 1H), 3.23 (d, J = 4.9 Hz, 1H), 2.98 (s, 3H), 2.65 – 2.55 (m, 2H), 2.54 – 2.43 (m, 1H), 2.13 (dq, J = 13.0, 6.9 Hz, 1H), 1.99 – 1.78 (m, 2H), 1.77 – 1.37 (m, 6H), 0.92 (d, J = 6.8 Hz, 3H), 0.83 (d, J = 6.5 Hz, 3H), 0.71 (t, J = 7.9 Hz, 3H), 0.59 (d, J = 6.8 Hz, 3H).

^{13}C NMR (125 MHz, MeOD): δ 173.9, 171.0, 170.7, 162.5, 157.2, 144.8, 137.1, 128.5, 128.0, 118.8, 66.7, 63.5, 56.7, 55.6, 54.6, 52.0, 43.3, 37.4, 36.0, 31.1, 30.8, 26.6, 24.5, 24.3, 20.8, 19.1, 14.8, 11.7.

5 Example 21: Hydrolysis of methyl ester: Synthesis of acid 27



LiOH (2.0 mmol) was added to a solution of **26** in a mixture of THF and H₂O (1:1, 0.5 M), and the reaction mixture was stirred at room temperature for 6 h. The mixture was concentrated under reduced pressure. The crude product was purified through silica gel column chromatography.

TLC: 0.25 (CH₂Cl₂/MeOH, 9:1)

Yield: 99%

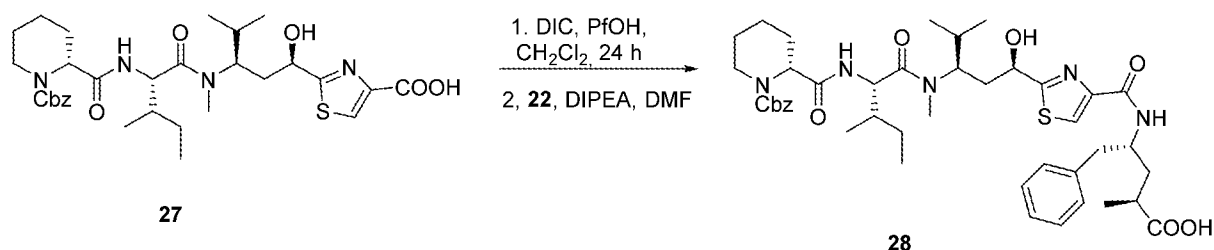
15 $[\alpha]_{\text{D}}^{25} = +18.5$ (C 1, MeOH)

MS (ESI) m/z calculated for C₃₁H₄₄N₄O₇S [M+H]⁺: 617.32, found 617.15

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.37 – 7.28 (m, 5H), 5.69 (d, J = 12.5 Hz, 1H), 5.35 – 5.22 (m, 2H), 4.94 (d, J = 7.0 Hz, 1H), 4.85 (t, J = 6.9 Hz, 1H), 4.20 (dt, J = 12.4, 7.0 Hz, 1H), 3.67 (ddd, J = 7.1, 3.8, 2.3 Hz, 1H), 3.47 (dt, J = 12.4, 7.0 Hz, 1H), 3.23 (d, J = 4.9 Hz, 1H), 2.95 (s, 3H), 2.80 (ddd, J = 12.4, 7.0, 2.4 Hz, 1H), 2.59 (dp, J = 13.7, 6.8 Hz, 1H), 2.38 (ddd, J = 12.3, 7.0, 3.8 Hz, 1H), 2.04 (dq, J = 12.7, 6.9 Hz, 1H), 1.95 – 1.78 (m, 2H), 1.78 – 1.66 (m, 1H), 1.66 – 1.55 (m, 2H), 1.52 – 1.39 (m, 2H), 1.26 (tq, J = 12.6, 8.0 Hz, 1H), 0.98 (d, J = 6.8 Hz, 3H), 0.82 (dd, J = 13.2, 6.8 Hz, 6H), 25 0.71 (t, J = 8.0 Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, MeOD): δ 173.9, 173.0, 171.0, 162.3, 157.2, 150.6, 137.1, 128.5, 128.0, 122.1, 66.7, 63.5, 56.7, 55.6, 54.6, 43.3, 37.4, 36.0, 31.1, 30.8, 26.6, 24.5, 24.3, 20.8, 19.1, 14.8, 11.7.

5 Example 22: Coupling of Tubuphenylalanine: Synthesis of 28



Acid **27** (1.0 mmol) was added to a 0.2 M solution of pentafluorophenol (1.25 mmol) and DIC (1.0 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was warmed to rt, stirred for 24 h, and concentrated under reduced pressure. EtOAc (10 mL) was added, and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated under reduced pressure, and the crude material was used without further purification. DMF (0.335 mL, 0.25 M) was added to the crude product, followed by the hydrochloride salt of tubuphenylalanine (**22**) (2.0 mmol) and diisopropylethylamine (4.0 mmol). The reaction mixture was stirred for 24 h at rt, and DMF was removed under vacuum. The crude product was purified by column chromatography (5% CH_2Cl_2 :MeOH) afforded **28** as white solid.

TLC: 0.32 (CH_2Cl_2 /MeOH/TFA, 9:1:0.1)

Yield: 90%

$[\alpha]_{\text{D}}^{25} = +75.2$ (C 1, MeOH)

MS (ESI) m/z calculated for $\text{C}_{43}\text{H}_{59}\text{N}_5\text{O}_8\text{S}$ $[\text{M}+\text{H}]^+$: 806.10, found 806.21

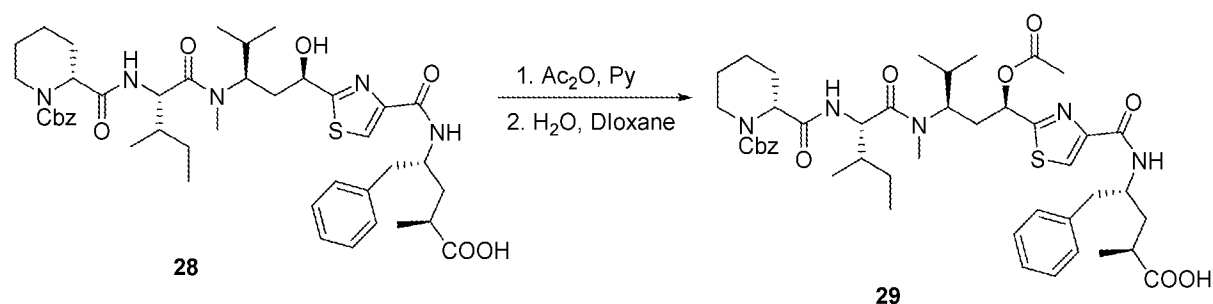
Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.38 – 7.24 (m, 7H), 7.24 – 7.13 (m, 3H), 6.04 (d, $J = 12.4$ Hz, 1H), 5.14 – 5.05 (m, 3H), 4.69 (t, $J = 6.9$ Hz, 1H), 4.33 (dt, $J = 12.5, 7.1$ Hz, 1H), 4.11 (dtd, $J = 11.5, 7.1, 1.0$ Hz, 1H), 3.57 (ddd, $J = 6.9, 4.2, 2.0$ Hz, 1H), 3.42 (dt, $J = 12.4, 7.0$ Hz, 1H), 3.23 (d, J

= 4.9 Hz, 1H), 3.15 (ddt, $J = 12.3, 6.9, 1.0$ Hz, 1H), 2.97 – 2.84 (m, 4H), 2.79 (ddd, $J = 12.4, 7.0, 2.0$ Hz, 1H), 2.69 – 2.61 (m, 1H), 2.61 – 2.53 (m, 2H), 2.36 (ddd, $J = 12.3, 7.0, 4.3$ Hz, 1H), 2.12 (td, $J = 11.7, 6.5$ Hz, 1H), 2.01 – 1.54 (m, 7H), 1.49 (dp, $J = 12.6, 6.8$ Hz, 1H), 1.34 – 1.20 (m, 4H), 0.94 (d, $J = 6.8$ Hz, 3H), 0.81 (dd, $J = 22.5, 6.8$ Hz, 6H), 0.71 (t, $J = 8.0$ Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, CDCl_3): δ 179.2, 173.9, 171.0, 167.6, 161.5, 157.2, 150.4, 137.1, 129.3, 128.5, 128.0, 126.3, 118.6, 66.7, 63.5, 56.7, 55.6, 54.6, 53.8, 43.3, 39.3, 37.6, 37.4, 36.0, 31.1, 30.8, 26.6, 24.5, 24.3, 20.8, 19.1, 18.3, 14.8, 11.7.

10

Example 23: Acetylation of hydroxyl group: Synthesis of 29



A 0.1 M solution of **28** (1.0 mmol) in pyridine (10.0 mmol) was cooled to 0 °C, and acetic anhydride (10.0 mmol) was added. The reaction mixture was allowed to warm to rt over 2 h and was stirred at rt for 24 h. The reaction mixture was then cooled to 0 °C, and a 1:1 mixture of dioxane/water (4 mL) was added. The mixture was allowed to warm to rt, and was stirred for 12 h at rt. The solvent was removed under reduced pressure. Column chromatography (100% CH_2Cl_2 to 10% $\text{MeOH}/\text{CH}_2\text{Cl}_2$) afforded **29** as an amorphous solid.

TLC: 0.50 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{TFA}$, 9:1:0.1)

Yield: 95%

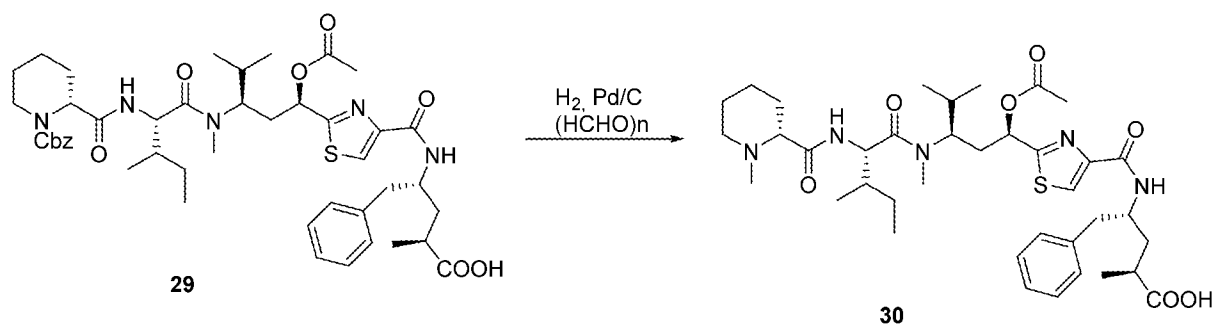
$[\alpha]_{\text{D}}^{25} = +26.5$ (C 1, MeOH)

MS (ESI) m/z calculated for $\text{C}_{45}\text{H}_{61}\text{N}_5\text{O}_9\text{S}$ $[\text{M}+\text{H}]^+$: 848.41, found 848.35

Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.38 – 7.24 (m, 7H), 7.24 – 7.13 (m, 3H), 5.68 (t, $J = 7.0$ Hz, 1H), 5.52 (d, $J = 12.4$ Hz, 1H), 5.42 – 5.35 (m, 1H), 4.92 (t, $J = 7.0$ Hz, 1H), 4.68 (d, $J = 6.9$ Hz, 1H), 4.24 (dt, $J = 12.6$, 7.1 Hz, 1H), 4.03 (ddd, $J = 12.6$, 7.0, 3.4 Hz, 1H), 3.92 (dtd, $J = 10.5$, 7.0, 1.8 Hz, 1H), 3.54 – 3.46 (m, 1H), 3.28 (dt, $J = 12.4$, 7.0 Hz, 1H), 3.09 (ddd, $J = 11.7$, 7.1, 3.4 Hz, 1H), 2.95 (s, 3H), 2.82 (ddt, $J = 12.4$, 7.0, 1.0 Hz, 1H), 2.59 (dp, $J = 13.6$, 6.8 Hz, 1H), 2.46 (h, $J = 6.9$ Hz, 1H), 2.20 – 2.12 (m, 5H), 2.10 – 1.93 (m, 3H), 1.93 – 1.82 (m, 1H), 1.77 – 1.51 (m, 4H), 1.39 (dp, $J = 13.0$, 7.0 Hz, 1H), 1.26 (d, $J = 6.8$ Hz, 3H), 1.17 (tq, $J = 13.0$, 8.2 Hz, 1H), 0.93 (d, $J = 6.8$ Hz, 3H), 0.77 – 0.67 (m, 9H).

Major rotamer: ^{13}C NMR (125 MHz, CDCl_3): δ 179.2, 173.9, 171.0, 170.5, 164.6, 161.5, 157.2, 150.4, 137.1, 129.3, 128.5, 128.0, 126.3, 118.6, 66.7, 62.5, 56.7, 55.6, 54.2, 53.8, 43.3, 39.3, 37.6, 37.2, 36.0, 35.8, 31.1, 30.8, 26.6, 24.5, 24.3, 21.3, 20.8, 19.1, 18.3, 14.8, 11.7.

Example 24: Cbz deprotection and N-methylation: Synthesis of 30



The compound **29** (1.0 mmol) was dissolved in a mixture of MeOH (20 ml). Paraformaldehyde (300 mg, 10 mmol) and 20% Pd/C (106 mg, 0.1 mmol Pd) were added. The reaction mixture was stirred under hydrogen atmosphere for 16 h and afterwards filtered through Cellite. The solvent was then removed under reduced pressure. The product was purified through column chromatography.

TLC: 0.51 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{TFA}$; 9:1:0.1).

Yield: 86%

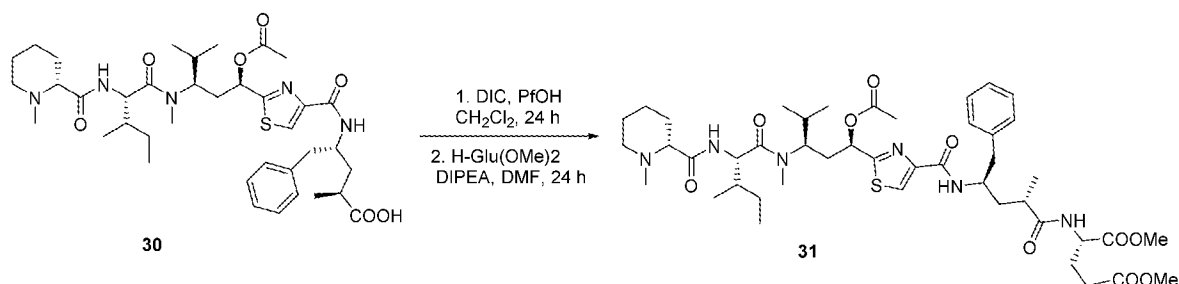
$[\alpha]_{\text{D}}^{25} = + 19.2$ (c=1.0, MeOH).

MS (ESI) m/z calculated for $\text{C}_{38}\text{H}_{57}\text{N}_5\text{O}_7\text{S}$ $[\text{M}+\text{H}]^+$: 728.40, found 728.35.

^1H NMR (500 MHz, MeOD): δ 8.08 (s, 1H), 7.19–7.25 (m, 4H), 7.13–7.18 (m, 1H), 5.71 (dd, 1H, $J = 2.5, 11.0$ Hz), 4.73 (d, 1H, $J = 8.0$ Hz), 4.30–4.50 (m, 2H), 3.10 (s, 3H), 3.05 (d, 1H, $J = 11.5$ Hz), 2.92 (d, 2H, $J = 6.5$ Hz), 2.85 (d, 1H, $J = 10.5$ Hz), 2.51 (br s, 1H), 2.23–2.41 (m, 3H), 2.31 (s, 3H), 2.15 (s, 3H), 1.96–2.05 (m, 1H), 1.75–1.92 (m, 4H), 1.56–1.74 (m, 5H), 1.37–1.41 (m, 1H), 1.09–1.23 (m, 1H), 1.16 (d, 3H, $J = 7.0$ Hz), 1.03 (d, 3H, $J = 6.5$ Hz), 0.98 (d, 3H, $J = 6.5$ Hz), 0.92 (t, 3H, $J = 7.3$ Hz), 0.81 (d, 3H, $J = 6.5$ Hz).

^{13}C NMR (125 MHz, MeOD): $\delta = 11.3, 16.4, 19.1, 20.4, 20.6, 20.9, 23.7, 25.5, 25.5, 30.9, 31.0, 31.1, 35.6, 37.6, 39.5, 39.6, 42.0, 44.2, 51.2, 55.2, 56.4, 69.7, 71.2, 125.1, 127.4, 129.3, 130.6, 139.8, 151.1, 162.7, 171.6, 171.8, 173.6, 175.0, 182.5$ ppm.

Example 25 : Synthesis of tubulysin conjugate.



Acid **30** (1.0 mmol) was added to a 0.2 M solution of pentafluorophenol (1.25 mmol) and DIC (1.0 mmol) in a CH_2Cl_2 at 0 °C. The reaction mixture was warmed to rt, stirred for 24 h, and concentrated under reduced pressure and dissolved in DMF (0.5 M). In another reaction vessel hydrochloride salt of H-Glu-(OMe)₂ (3.0 mmol) and diisopropylethylamine (5.0 mmol) were dissolved in DMF (0.335 mL, 0.25 M) and this solution is added to the above prepared pentafluorophenyl ester. The reaction mixture was stirred for 24 h at rt, and DMF was removed under vacuum. The crude product was purified by RP-

semi HPLC (20-80% CH₃CN in H₂O for 30 min at 214 nm) afforded **31** as white off solid.

HPLC analysis: t_R = 3.44 min (Chromolith, gradient: 20-70% B/A over 5 min)

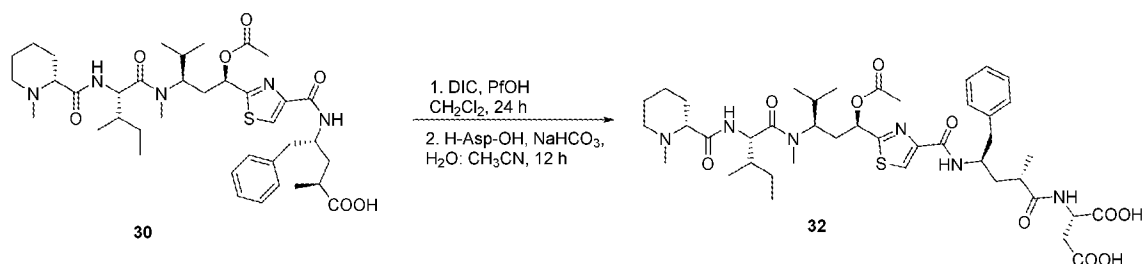
- 5 HPLC purification: Nucleosil C18, gradient: 20-21% B/A over 12 min, 25 °C
Yield: 75%

$[\alpha]_D^{25} = +75.2$ (C 1, MeOH)

MS (ESI) m/z calculated for C₄₅H₆₈N₆O₁₀S [M+Na]⁺: 907.46, found 907.40

- Major rotamer: ¹H NMR (500 MHz, MeOD): δ 7.41 – 7.16 (m, 7H), 7.12 –
10 7.00 (m, 3H), 6.98 (d, J = 18.1 Hz, 1H), 6.04 (d, J = 12.4 Hz, 1H), 5.55 (br, s,
1H), 5.25 – 5.20 (m, 3H), 4.25 (t, J = 6.9 Hz, 1H), 4.23 – 4.10 (m, 3H), 3.65 (s,
3H), 3.61 (s, 3H), 3.57 – 3.55 (m, 1H), 3.42 (dt, J = 12.4, 7.0 Hz, 1H), 3.23 (d,
 J = 4.9 Hz, 1H), 3.15 (ddt, J = 12.3, 6.9, 1.0 Hz, 1H), 3.10 – 3.00 (m, 2H),
2.97 – 2.84 (m, 4H), 2.79 – 2.72 (m, 3H), 2.69 – 2.61 (m, 1H), 2.61 – 2.53 (m,
15 2H), 2.36 (ddd, J = 12.3, 7.0, 4.3 Hz, 1H), 2.12 (td, J = 11.7, 6.5 Hz, 1H), 2.01
– 1.54 (m, 7H), 1.49 (d, J = 12.6, 6.8 Hz, 1H), 1.34 – 1.20 (m, 4H), 0.94 (d, J =
6.8 Hz, 3H), 0.81 (dd, J = 22.5, 6.8 Hz, 6H), 0.71 (t, J = 8.0 Hz, 3H).

- Major rotamer: ¹³C NMR (125 MHz, CDCl₃): δ 180.2, 179.4, 178.2, 178.0,
173.0, 171.5, 166.3, 161.9, 157.0, 150.5, 137.4, 129.0, 128.2, 128.0, 126.1,
20 118.1, 66.1, 63.7, 56.7, 55.5, 54.5, 53.0, 48.8, 45.5, 43.0, 39.7, 37.9, 36.5, 36.1,
31.1, 30.8, 26.7, 24.0, 24.1, 20.7, 19.1, 18.3, 14.8, 12.5, 11.9, 11.2.

Example 26 : Synthesis of tubulysin conjugate

- 5 Acid **30** (1.0 mmol) was added to a 0.2 M solution of pentafluorophenol (1.25 mmol) and DIC (1.0 mmol) in a CH_2Cl_2 at 0 °C. The reaction mixture was warmed to rt, stirred for 24 h, and concentrated under reduced pressure and dissolved in acetonitrile (0.5 M). In another reaction vessel (L) H-Asp-OH, (2.0 mmol) and sodium bicarbonate (4.0 mmole) were dissolved in 2 ml. of
- 10 water and adjust the pH about 8. Then a solution of pentafluorophenyl ester prepared above (1.0 mmole) 3 mL of acetonitrile was added at room temperature. The reaction mixture was stirred overnight. The solvent were removed under vacuum. The crude product was purified by RP-semi HPLC (20-60% CH_3CN in H_2O for 30 min at 214 nm) afforded **32** as white off solid.
- 15 HPLC analysis: $t_R = 2.82$ min (Chromolith, gradient: 20-60% B/A over 5 min)

HPLC purification: Nucleosil C18, gradient: 20-60% B/A over 30 min

Yield: 68 %

$[\alpha]_{\text{D}}^{25} = + 125.6$ (C 1, MeOH)

- 20 MS (ESI) m/z calculated for $\text{C}_{42}\text{H}_{62}\text{N}_6\text{O}_{10}\text{S}$ $[\text{M}+\text{H}]^+$: 842.42, found 842.40
- Major rotamer: ^1H NMR (500 MHz, MeOD): δ 7.38 – 7.19 (m, 7H), 7.10 – 6.89 (m, 3H), 6.80 (d, $J = 18.1$ Hz, 1H), 6.55 (d, $J = 12.4$ Hz, 1H), 5.89 (br, s, 1H), 5.11 – 5.00 (m, 3H), 4.25 (t, $J = 6.9$ Hz, 1H), 4.32 – 4.13 (m, 3H), 3.55 – 3.38 (m, 1H), 3.40 (dt, $J = 12.4, 7.0$ Hz, 1H), 3.23 (d, $J = 4.9$ Hz, 1H), 3.15
- 25 (ddt, $J = 12.3, 6.9, 1.0$ Hz, 1H), 3.10 – 2.90 (m, 2H), 2.80 – 2.71 (m, 2H), 2.66 – 2.62 (m, 3H), 2.55 – 2.45 (m, 1H), 2.40 – 2.33 (m, 2H), 2.30 (ddd, $J = 12.3, 7.0, 4.3$ Hz, 1H), 2.10 (td, $J = 11.7, 6.5$ Hz, 1H), 1.96 – 1.64 (m, 7H), 1.52 (d,

$J = 12.6, 6.8$ Hz, 1H), 1.41 – 1.30 (m, 4H), 0.94 (d, $J = 6.8$ Hz, 3H), 0.81 (dd, $J = 22.5, 6.8$ Hz, 6H), 0.71 (t, $J = 8.0$ Hz, 3H).

Major rotamer: ^{13}C NMR (125 MHz, CDCl_3): δ 182.6, 180.1, 177.1, 172.0, 171.6, 165.3, 160.9, 155.2, 150.8, 138.2, 128.0, 127.2, 127.4, 126.2, 118.4,
5 65.8, 59.7, 55.7, 53.4, 53.7, 48.6, 45.0, 43.7, 38.1, 37.7, 37.5, 35.7, 31.0, 30.2, 26.7, 25.8, 24.1, 21.5, 19.8, 18.0, 13.6, 12.1, 11.0.

It was found that compound **30** (100 nM test concentration) is highly toxic to HeLa cells after 18hrs treatment, thus demonstrating its suitability as
10 cytotoxic agent e.g. in targeted drug delivery.

Example 27: Further tubulysin derivatives

A person skilled in the art will understand and appreciate that the present
15 invention relies on two parts. One part is the Passerini MCR- based approach to construct a tetrapeptide adduct in a good yield using only three synthetic steps.

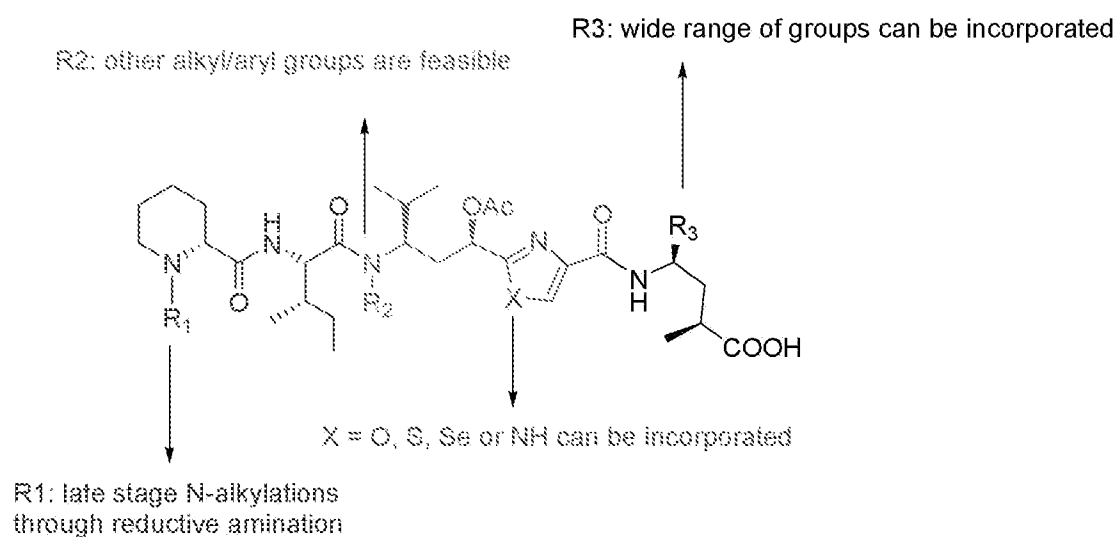
Secondly, as is exemplified by the synthesis of Tubuphenylalanine (Tup;
20 general formula G), the invention uses simple reagents and less steps to obtain Tup in a highly diastereoselective manner. Another major advantage of this method is that, any other unnatural γ -amino acids are easily obtainable by the simple Grignard reaction. This diversity has never been studied so far.

25 The tubulysin “derivative” is therefore considered in the broad context and allows for:

- Variation on the *N*-alkylated Tuv substituents (Not only limited to *N*-Me),
 - Diversity possible from the X-substituents from the isocyanide
- 30

- Higher alkyl chains (not only *N*-Me) can be incorporated on the N-terminal Picolinic acid
- Wide range of Tup derivatives are also constructed

5 See the following scheme:

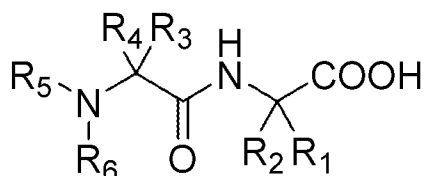


10 All these variations can be easily adopted through the standard protocol as herein disclosed, thus allowing to obtain a wide range of tubulysin derivatives.

Claims

1. A method for preparing a tubulysin derivative, comprising reacting compounds A, B and C in a 3-component Passerini reaction,

wherein compound A is a carboxylic acid according to the general
5 formula A



wherein

10 R_1 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; or a substituted or unsubstituted benzyl,

R_2 represents H, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl;

15 R_3 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; or a substituted or unsubstituted benzyl;

R_4 represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, a substituted or
20 unsubstituted benzyl;

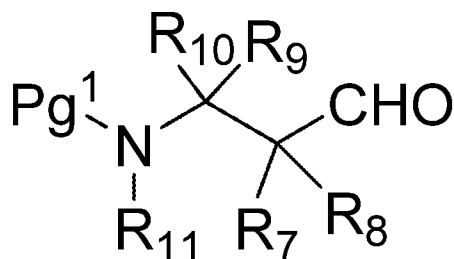
R_5 represents a substituted or unsubstituted alkyl or a substituted or unsubstituted cycloalkyl; preferably a substituted or unsubstituted cycloalkyl;

25 or wherein R_4 and R_5 are connected to form a 4- to 7-membered ring;

R_6 represents a substituted or unsubstituted alkyl; a substituted or unsubstituted cycloalkyl; a substituted or unsubstituted benzyl, or $C=OOR'$, where R' is an optionally substituted alkyl, cycloalkyl, benzyl or aroyl moiety;

5

wherein compound B is an aldehyde according to the general formula B



10

wherein R_7 , R_8 , R_9 , R_{10} and R_{11} each independently represent H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl or a substituted or unsubstituted benzyl;

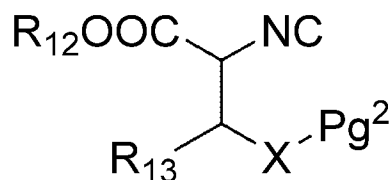
15

Pg^1 is an amine protecting group, preferably a carbamate, a substituted or an unsubstituted benzyl, or a substituted or an unsubstituted sulfonamide;

and

20

wherein compound C is an isocyanide according to the general formula C



wherein

R_{12} represents a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

25

R_{13} represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

X represents O, S, Se, or -NH-, preferably S; and

5 Pg^2 represents an X- protecting group, preferably selected from trityl, tert-butyl, adamantyl and substituted benzyl, more preferably trityl or tert-butyl.

2. Method according to claim 1, wherein

10 R_1 represents isopropyl, tert-butyl, iso-butyl, sec-butyl, cyclopropylmethyl or cyclobutylmethyl;

R_2 is H;

R_3 is $-(CH_2)_n-CH_3$, wherein n is 3 to 5;

R_4 is CH_2-CH_2- or $-CH_2-CH_2-CH_2-$ connected to R_5 ;

15 R_5 is a substituted or unsubstituted cycloalkyl; and/or

R_6 is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate and ethyl carbamate.

3. Method according to claim 1 or 2, wherein

R_7 is H;

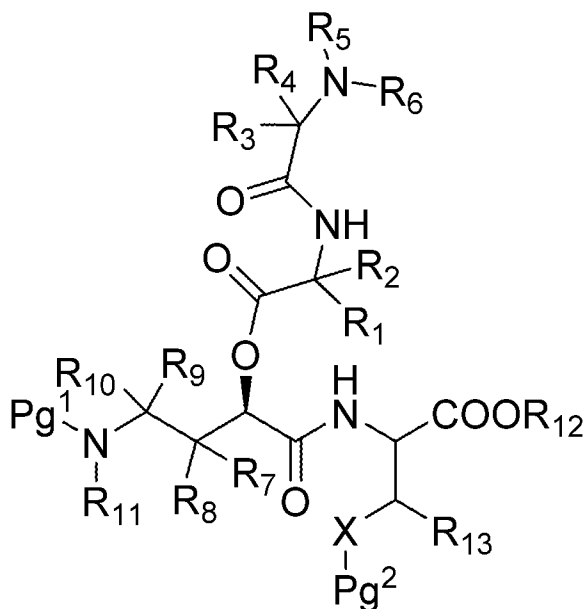
25 R_8 is H;

R_9 is selected from the group consisting of isopropyl, cyclopropyl, cyclobutyl, isobutyl, sec-butyl, tert-butyl and cyclopropylmethyl;

R_{10} is H; and/or

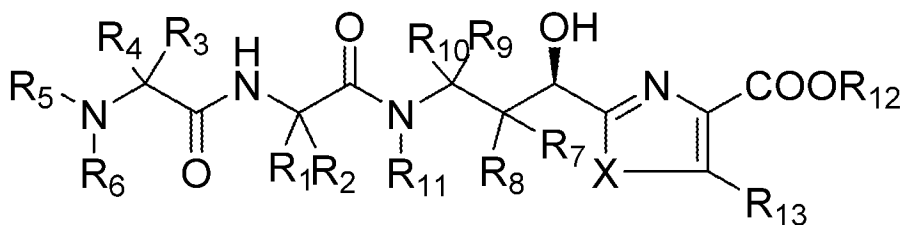
30 R_{11} is selected from the group consisting of methyl, ethyl, propyl, butyl, isopropyl, cyclopropyl and cyclopropylmethyl.

4. Method according to any one of the preceding claims, wherein Pg^2 is selected from the group consisting of benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl.
- 10 5. Method according to any one of the preceding claims, wherein R_{12} is methyl, ethyl or tert-butyl; and/or wherein R_{13} is H or methyl.
6. Method according to any one of the preceding claims, comprising reacting compounds A, B and C in a non-coordinating solvent or solvent mixture, preferably selected from the group consisting of CH_2Cl_2 , CHCl_3 , CCl_4 , benzene, THF, CH_3CN , 1,4-dioxane, 1,2-dichloroethane and a mixture of CH_2Cl_2 :THF (1:1 v/v).
- 15
7. Method according to any one of the preceding claims, further comprising isolating the 3-component Passerini reaction product of Formula D
- 20 D



wherein each of the substituents is as defined in claims 1-5.

8. Method according to claim 7, further comprising subjecting the Passerini reaction product of formula D to (a) an acyl migration reaction and (b) a cyclodehydration reaction of the Cys-amide to obtain a compound of the general formula E



9. Method according to claim 8, wherein said acyl migration reaction is performed in a two-step process involving exposure to diethylamine (DEA) followed by exposure to trimethylamine (TEA).

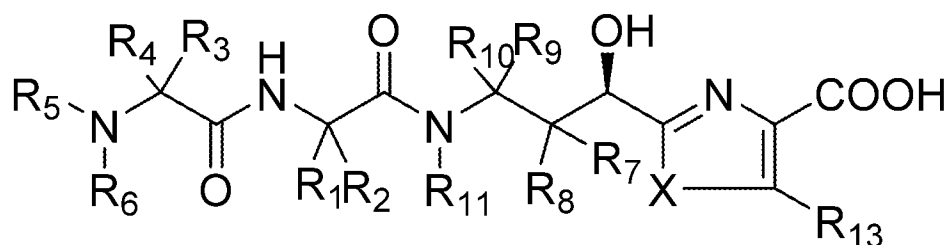
10. Method according to claim 8 or 9, wherein said cyclodehydration reaction is performed in a two-step process involving incubation in the

presence of TiCl_4 , followed by oxidation in the presence of MnO_2 , preferably activated MnO_2 having a pore size of ≤ 5 microns.

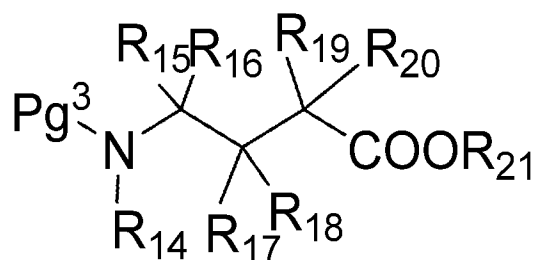
11. Method according to any one of claims 8-10, wherein acyl
5 migration precedes cyclodehydration.

12. Method according to any one of claims 8-10, wherein
cyclodehydration precedes acyl migration.

10 13. Method according to any one of claims 8 to 12, further comprising
hydrolyzing the ester of the general formula E to obtain the carboxylic acid
compound of the general formula F



15 14. Method according to claim 13, further comprising reacting the
carboxylic acid compound of the formula F with a compound of the general
formula G



20

wherein

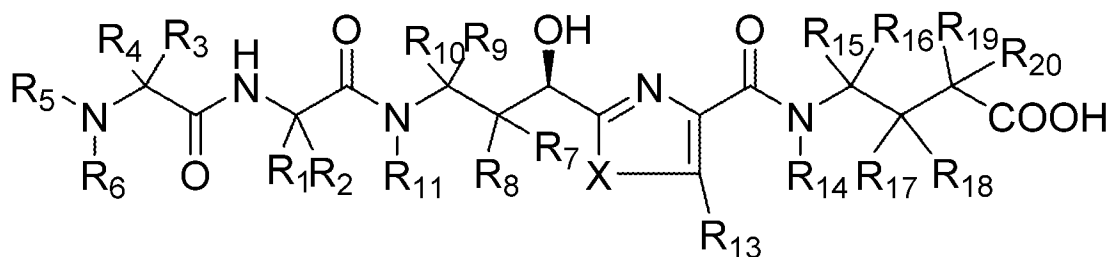
R_{14} , R_{15} , R_{16} , R_{20} and R_{21} each independently represent H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;

R_{17} and R_{18} each independently represent H, F, a substituted or unsubstituted alkyl, or a substituted or unsubstituted cycloalkyl;

R_{19} represents H, F, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl; and

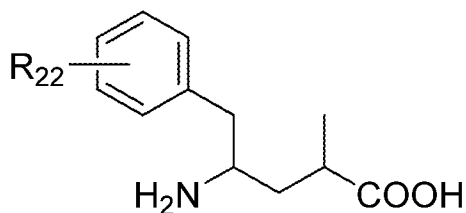
Pg^3 is an amine protecting group, preferably a carbamate, a substituted or unsubstituted benzyl, preferably selected from the group consisting of tertbutyl sulfine, benzyloxycarbonyl, 4-azidobenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, 4,5-dimethoxy-2-nitrobenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 1-naphthylmethoxycarbonyl, 4-acetyloxybenzyloxycarbonyl, fluorenyloxycarbonyl, tert-butyloxycarbonyl, allyloxycarbonyl, methyl carbamate, ethyl carbamate, benzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl

followed by removal of protecting moiety R_{21} ,
to obtain a compound of the general formula H



20

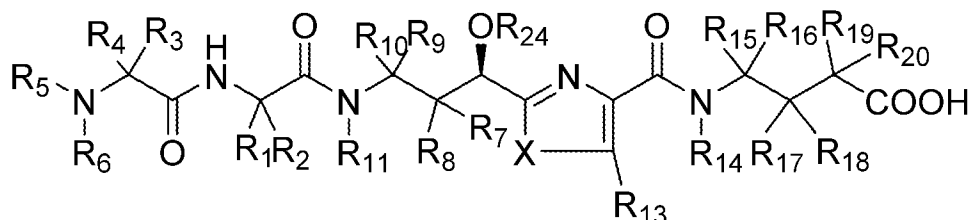
15. Method according to claim 14, wherein the compound of the general formula G is tubuphenylalanine of the formula.



or a salt thereof,

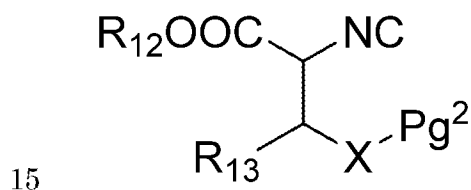
wherein R_{22} is H, OH, F or NO_2 , preferably H, OH or F.

16. Method according to claim 14 or 15, further comprising the steps of acylation of the hydroxyl group of the general formula H to obtain a
5 compound of the general Formula I



- wherein R_{24} represent acetyl, acyl (substituted) alkyl, acyl cycloalkyl, or
10 acyl benzyl, preferably acetyl or acyl derivative of methyl, ethyl, tert-butyl or benzyl.

17. An isocyanide compound according to the general formula C

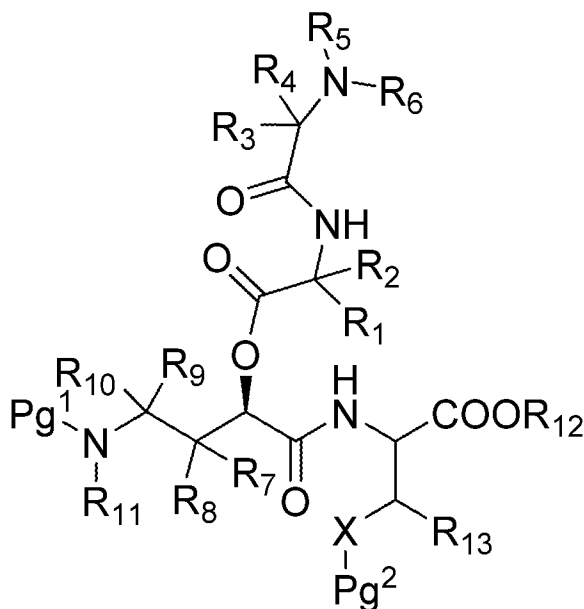


wherein

- R_{12} represents a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;
20 R_{13} represents H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted benzyl;
 X represents O, S, Se, or -NH- , preferably S; and

Pg² represents a -X protecting group, preferably selected from the group consisting of trityl, tert-butyl, adamantly, and a substituted benzyl, more preferably trityl or tert-butyl.

- 5 18. The 3-component Passerini reaction product obtainable by the
method of claim 7, having the general formula



wherein each of the substituents is as defined in claims 1-5.

- 10
19. The use of an isocyanide compound according to claim 17 in the manufacture of a tubulysin derivative.
20. The use of the Passerini reaction product according to claim 18 in
15 the manufacture of a tubulysin derivative.
21. The use according to claim 19 or 20, in the manufacture of a tubulysin-prodrug or a tubulysin ADC.

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2019/050481

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K5/02 A61K47/54 A61K47/68 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, EMBASE, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THIMMALAPURA M. VISHWANATHA ET AL: "Cysteine Isocyanide in Multicomponent Reaction: Synthesis of Peptido-Mimetic 1,3-Azoles", JOURNAL OF ORGANIC CHEMISTRY, vol. 82, no. 18, 25 August 2017 (2017-08-25), pages 9585-9594, XP055544660, ISSN: 0022-3263, DOI: 10.1021/acs.joc.7b01615 compound 4 ----- -/--	17
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
6 November 2019		13/11/2019
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer
		Monami, Amélie

INTERNATIONAL SEARCH REPORT

International application No

PCT/NL2019/050481

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	----- TAKUYA FUKUDA ET AL: "Anti-barnacle Activity of Isocyanides Derived from Amino Acids", CHEMISTRY & BIODIVERSITY, vol. 13, no. 11, 1 November 2016 (2016-11-01), pages 1502-1510, XP055544849, CH ISSN: 1612-1872, DOI: 10.1002/cbdv.201600063 compound 6	17
A	----- EP 2 409 983 A1 (LEIBNIZ INST FUER PFLANZENBIOCHEMIE IPB [DE]) 25 January 2012 (2012-01-25) paragraph [0007] - paragraph [0009] paragraph [0014] - paragraph [0015] figures 1, 2	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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