Biochemical Investigations on Microbial Prenyltransferases in the Presence of DMAPP Analogues

Biochemische Untersuchungen an mikrobiellen Prenyltransferasen in Anwesenheit von DMAPP-Analoga

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Mike Patrick Liebhold
aus Dresden

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Erstgutachter: **Prof. Dr. Shu-Ming Li**
Zweitgutachter: **Prof. Dr. Michael Keusgen**

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Dedicated to

my family

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Julia Winkelblech*, **Mike Liebhold***, Xiulan Xie and Shu-Ming Li (2014). Unnatural DMAPP Analogues turn C-6 into the Preferable Alkylation/Benzylation Position for Tryptophan C5-, C6- and C7-Prenylating Enzymes. *Organic Letters* (submitted).

Mike Liebhold and Shu-Ming Li (2013). Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase. *Organic Letters* 15: 5834–5837.

Mike Liebhold, Xiulan Xie and Shu-Ming Li (2013). Breaking Cyclic Dipeptide Prenyltransferase Regioselectivity by Unnatural Alkyl Donors. *Organic Letters* 15: 3062–3065.

Mike Liebhold, Xiulan Xie and Shu-Ming Li (2012). Expansion of Enzymatic Friedel–Crafts Alkylation on Indoles: Acceptance of Unnatural β-unsaturated Allyl Diphospates by Dimethylallyl-tryptophan Synthases. *Organic Letters* 14: 4882–4885.

Xia Yu, Georg Zocher, Xiulan Xie, **Mike Liebhold**, Stefan Schütz, Thilo Stehle, and Shu-Ming Li (2013). Catalytic Mechanism of Stereospecific Formation of *cis*-Configured Prenylated Pyrroloindoline Diketopiperazines by Indole Prenyltransferases. *Chemistry and Biology* 20: 1492–1501.

Jan Michael Schuller, Georg Zocher, **Mike Liebhold**, Xiulan Xie, Mark Stahl, Shu-Ming Li and Thilo Stehle (2012). Structure and Catalytic Mechanism of a Cyclic Dipeptide Prenyltransferase with Broad Substrate Promiscuity. *Journal of Molecular Biology* 422:87–99.

* these authors contributed equally to this work

Share of author contributions

Publication	Authors	Estimated equity ratio [%]
Expansion of Enzymatic Friedel–Crafts Alkylation on Indoles: Acceptance of Unnatural β-Unsaturated Allyl Diphospates by Dimethylallyl-tryptophan Synthases (published)	Liebhold, M.; Xie, X.; Li, S.-M.	70
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Oral and poster presentations

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Expansion of Friedel–Crafts alkylation on indoles by using unnatural allyl diphosphates

Short lecture and poster presentation, 25. Irseer Naturstofftage (Dechema), 20.–22. February 2013, Kloster Irsee

Mike Liebhold, Xiulan Xie and Shu-Ming Li

Breaking of the regioselectivity of several prenyltransferases by non-natural alkyl donors

Poster presentation, 1. European Conference on Natural Products: Research and Applications (Dechema), 22.–25. September 2013, Frankfurt

Abbreviations

For units of measurements, the international system of units (SI; Le Système international d'unités) and units derived thereof have been used. Acronyms and abbreviations are explained in detail in the corresponding passage in the text.

2-pen-PP	2-pentenyl diphosphate
2-pentenyl-PP	2-pentenyl diphosphate (also *trans*-pentenyl diphosphate)
3-methylbutanyl-PP	3-methylbutanyl diphosphate
4-methyl-3-pentenyl-PP	4-methyl-3-pentenyl diphosphate
5-DMATS	5-dimethylallyl tryptophan synthase
5-DMATS$_{Sc}$	5-dimethylallyl tryptophan synthase from *Streptomyces coelicolor*
6-DMATS	6-dimethylallyl tryptophan synthase
6-DMATS$_{Sa}$	6-dimethylallyl tryptophan synthase from *Streptomyces ambofaciens*
6-DMATS$_{Sv}$	6-dimethylallyl tryptophan synthase from *Streptomyces violaceusniger*
7-DMATS	7-dimethylallyl tryptophan synthase
2D NMR	2 dimensional nuclear magnetic resonance
A. clavatus	*Aspergillus clavatus*
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. terreus	*Aspergillus terreus*
A. versicolor	*Aspergillus versicolor*
AGPP	anilinogeranyl diphosphate
alkyl-PP	alkyl diphosphate
benzyl-PP	benzyl diphosphate
br	broad (NMR signal)
C. purpurea	*Claviceps purpurea*
CD_3OD	deuterated methanol
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CoA	Coenzyme A
Comp.	compound

Conc.	concentration
COSY	correlation spectroscopy
Cyclo-L-Trp-L-Leu	*cyclo*-L-tryptophanyl-L-leucinyl
Cyclo-L-Trp-L-Pro	*cyclo*-L-tryptophanyl-L-prolinyl
δ_H	proton chemical shift
δ_C	carbon chemical shift
D_2O	deuterium oxide
d	doublet
DCM	dichloromethane
DMA	dimethylallyl
DMAPP	dimethylallyl diphosphate
DMAT	dimethylallyl tryptophan
DMATS	dimethylallyl tryptophan synthase
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxid
DMSO-*d6*	deuterated dimethyl sulfoxid
DNA	deoxyribonucleic acid
DXP	1-deoxy-D-xylulose 5-phosphate
E. coli	*Escherichia coli*
EI-MS	electron impact mass spectrometry
ESI-MS	electrospray ionization spectrometry
FPP	farnesyl diphosphate
GPP	geranyl diphosphate
GGPP	geranylgeranyl diphosphate
HCl	hydrogen chloride
His_5	pentahistidine
His_6	hexahistidine
His_8	octahistidine
HMBC	heteronuclear multiple bond correlation
HMG	3-hydroxy-3-methylglutaryl
HPLC	high performance liquid chromatography
HR	high resolution
HSQC	heteronuclear single quantum coherence
Hz	hertz
IPP	isopentenyl diphosphate
IPTG	isopropyl β-thiogalactopyranoside

J	coupling constant
k_{cat}	turnover number
kDa	kilo Dalton
K_M	Michaelis-Menten constant
L. maculans	*Leptosphaeria maculans*
L	liter
L-Trp	L-tryptophan
LB	lysogeny broth
m	multiplet
MAPP	monomethylallyl diphosphate (also methylallyl diphosphate)
MEP	2-*C*-methyl-D-erythritol 4-phosphate
MeOH	methanol
MHz	mega hertz
MTase	methyltransferase
MVA	mevalonate
MS	mass spectrometry
multi	multiplicity
n × C-5	number of C-5 units, minimum 1
N. fischeri	*Neosatorya fischeri*
NCS	*N*-chlorosuccinimide
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
NRPS	non-ribosomal peptide synthase
OD_{600}	optical density at 600 nm
P. aethiopicum	*Penicillium aethiopicum*
P. roqueforti	*Penicillium roqueforti*
Ph. amygdali	*Phomopsis amygdali*
PMTase	protein methyltransferases
PPi	inorganic diphosphate
ppm	parts per million
q	quartet
rel. conv.	relative conversion
RP	reverse phase
rpm	revolutions per minute
S. ambofaciens	*Streptomyces ambofaciens*

S. cinnamonensis	*Streptomyces cinnamonensis*
S. coelicolor	*Streptomyces coelicolor*
S. violaceusniger	*Streptomyces violaceusniger*
Sa. cerevisiae	*Saccharomyces cerevisiae*
s	singlet
SAM	*S*-adenosyl-L-methionine
SeAM	*Se*-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp.	species (sing.)
spp.	species (pl.)
syn.	synonymous
t	triplet
TB	Terrific-Broth
TBA	tetrabutylammonium
Tris	2-amino-2-(hydroxymethyl)-propan-1,3-diol
v/v	volume per volume
$[M]^+$	molecular ion
$[M+H]^+$	molecular ion plus hydrogen
$[M-Na]^+$	molecular ion plus sodium
× g	gravitational acceleration

Summary

Plants, bacteria and fungi provide a plethora of diverse structures derived from the primary as well as the secondary metabolism. Representative substances from secondary metabolite pathways are flavonoids, coumarins, xanthones and indole alkaloids. Although not necessary for the growth and reproduction of the respective organisms, nevertheless these compounds give them an advantage over other organisms in the form of, e.g. attractants or protection agents against natural enemies as well as competitors. The attachment of isoprene units (n × C-5) such as dimethylallyl, geranyl or farnesyl moieties to aromatic secondary metabolites as backbones is a further step in the broad diversification of these compounds. Enzymes of the class of prenyltransferases accomplish this prenyl transfer reaction in nature. The prenylated natural products often exhibit strong pharmacological activities in contrast to their substrates. Therefore, prenylated compounds represent interesting targets for drug development. Investigations on alternative alkyl residues transferable via prenyltransferases, i.e. prenyl diphosphate analogues, could have the potential to play an important role in the understanding of the catalytic mechanism of these enzymes as well as in the finding process of new drugs.

As the first project in this thesis, the acceptance of four chemically synthesized alkyl and allyl diphosphates in the presence of three L-tryptophan prenyltransferases (FgaPT2, 5-DMATS, 7-DMATS) was elucidated. Retaining the double bond in β-position and performing alterations on the methyl groups of the allyl residue led to regular alkylated products and thereby showed successful utilization of DMAPP analogues. However, depending on the DMAPP analogue, the regiospecificity of the applied prenyltransferases was shifted partially or completely. Furthermore, the enzymes did not catalyze the transfer reaction of alkyl diphosphates onto the L-tryptophan scaffold, if no double bond was present or if it was relocated to γ-position compared to DMAPP.

Subsequently, the behavior of cyclic dipeptide prenyltransferases (AnaPT, CdpNPT, CdpC3PT, FtmPT1, BrePT) towards the unnatural alkyl and allyl donors was examined. The cyclic dipeptide prenyltransferases also used the unsaturated DMAPP analogues resulting in the formation of multiple products. Regardless of the prenylation position and orientation for the prenyl attachment (C-2 or C-3; regular or reverse) in the presence of DMAPP, the enzymatic reactions with both DMAPP analogues, i.e. MAPP and 2-pentenyl-PP, yielded a mixture of C2-reverse as well as C3-reverse alkylated diastereomers in different ratios depending on the donor and used enzyme.

After the successful alkylation of L-tryptophan and tryptophan-containing cyclic dipeptides utilizing simple unnatural allyl DMAPP analogues, the acceptance of the structurally more complex DMAPP analogue benzyl diphosphate was tested. Preliminary investigations showed the successful usage of this benzyl donor by several L-tryptophan and cyclic dipeptide prenyltransferases, whereas FgaPT2 showed the highest activity. FgaPT2 also displayed a remarkable promiscuity using tryptophan analogues as substrates and catalyzing a highly regiospecific C5-benzylation. Consequently, the usage of benzyl diphosphate instead of dimethylallyl diphosphate results in a complete shift of the prenylation position from C-4 to C-5.

To complete our findings regarding the acceptance of unnatural alkyl and benzyl analogues by prenyltransferases, several tryptophan C5-, C6- and C7-prenylating enzymes of fungal and bacterial origin have been assayed with the three analogues. Depending on the enzyme used, one to four products could be identified from the incubation mixtures. The predominant products were regular C6-alkylated or C6-benzylated derivatives in all cases. Therefore, for the tested tryptophan C5-, C6- and C7-prenylating enzymes, C-6 seemed to be the preferred position for attachment of the alkyl or benzyl moiety.

The results obtained during this thesis show that allyl as well as benzyl analogues of DMAPP are potential alternatives for chemoenzymatic Friedel–Crafts alkylations of simple indole derivatives and tryptophan-containing cyclic dipeptides and could be used for the production of alkylated compounds.

Zusammenfassung

Pflanzen, Bakterien und Pilze weisen eine Fülle an unterschiedlichen Strukturen sowohl aus dem Primär- als auch aus dem Sekundärstoffwechsel auf. Vertreter, der aus dem Sekundärstoffwechsel stammenden Substanzen, sind unter anderem Flavonoide, Coumarine, Xanthone und Indol-Alkaloide. Wenngleich sie nicht essenziell für das Wachstum und die Reproduktion der entsprechenden Organismen sind, verschaffen diese Stoffe ihren Produzenten dennoch einen Vorteil gegenüber anderen Organismen, zum Beispiel in Form von Lockstoffen, als Fraßschutz sowie als Abwehrstoffe gegen Konkurrenten. Das Anbringen von Isopren-Einheiten (n × C5) wie zum Beispiel Dimethylallyl-, Geranyl- oder Farnesyl-Gruppen an diese Grundstrukturen aus dem Sekundärstoffwechsel ist ein weiterer Schritt zur breiten Differenzierung dieser Stoffe. In der Natur bewerkstelligen Prenyltransferasen diese Prenylierungsreaktion. Prenylierte Naturstoffe weisen, im Gegensatz zu ihren Substraten, oftmals starke pharmakologische Wirkungen auf. Daher stellen sie interessante Moleküle für die Entwicklung von Arzneistoffen dar. Untersuchungen an, mit Hilfe von Prenyltransferasen übertragbaren, alternativen Alkyl-Resten, sogenannten Prenyl-Diphosphat Analogen, könnten eine wichtige Rolle beim Verstehen der katalytischen Mechanismen dieser Enzyme und ebenso bei der Entwicklung neuer Arzneistoffe spielen.

Zu Beginn dieser Dissertation wurde die Akzeptanz vier chemisch synthetisierter Alkyl- und Allyl-Diphosphate durch drei L-Tryptophan-Prenyltransferasen (FgaPT2, 5-DMATS, 7-DMATS) aufgeklärt. Wurde die Doppelbindung an β-Position beibehalten und nur die Methylgruppen verändert, so führte dies zur Bildung von regulär alkylierten Produkten und zeigten damit die erfolgreiche Anwendung von DMAPP-Analoga. Es wurde jedoch eine teilweise bzw. vollständige Verschiebung der Regiospezifität der verwendeten Prenyltransferasen beobachtet, abhängig vom eingesetzten DMAPP Analog. Die Enzyme katalysierten hingegen nicht die Übertragung von Alkyl-Diphosphaten mit fehlender oder bzw. nach γ-Position verschobener Doppelbindung.

Weiterhin wurde das Verhalten zyklischer Dipeptid-Prenyltransferasen (AnaPT, CdpNPT, CdpC3PT, FtmPT1, BrePT) gegenüber unatürlichen Alkyl- und Allyl-Donoren untersucht. Die zyklischen Dipeptid-Prenyltransferasen akzeptierten ebenfalls die ungesättigten DMAPP-Analoga MAPP und 2-pentenyl-PP und führten zur Bildung mehrerer Produkte. Ungeachtet der Position und Ausrichtung des Prenyl-Restes (C-2 oder C-3) in Anwesenheit von DMAPP, brachten die Reaktionen dieser Enzyme eine Mischung von C2- sowie C3-revers

alkylierten Diastereomeren in unterschiedlichen Verhältnissen hervor, abhängig vom gewählten Donor sowie Enzym.

Nach der erfolgreichen Alkylierung von L-Tryptophan sowie Tryptophan-haltigen zyklischen Dipeptiden mittels einfacher allylischer DMAPP-Analoga wurde die Akzeptanz eines strukturell komplexeren DMAPP-Analogons (Benzyl-Diphosphat) getestet. Vorangegangene Untersuchungen zeigten, dass mehrere L-Tryptophan- sowie zyklische Dipeptid-Prenyltransferasen diesen Benzyl-Donor akzeptierten, wobei FgaPT2 den höchsten Umsatz verzeichnete. FgaPT2 wies außerdem eine breite Substratspezifität auf und nutzte sowohl L-Tryptophan als auch verschiedene Derivate hiervon. In diesen Fällen katalysierte diese Prenyltransferase mit hoher Regiospezifität eine C5-Benzylierung, was eine vollständige Verschiebung der Prenylierungposition von Position C-4 darstellt.

Um die Untersuchungen bezüglich der Akzeptanz nicht natürlicher Alkyl- sowie Benzyl-Analoga abzurunden, wurden verschiedene Enzyme, welche Tryptophan an den Positionen C-5, C-6 oder C-7 prenylieren, aus Pilzen wie auch aus Bakterien zusammen mit den drei DMAPP Analoga inkubiert. Je nach verwendetem Enzym konnten bis zu vier Produkte isoliert werden. In allen Enzymassays war C6-alkyliertes oder benzyliertes L-Tryptophan das Hauptprodukt. Position C-6 des Indolringes wurde somit für die C-5, C-6 oder C-7 prenylierenden Enzyme zur bevorzugten Angriffspunkt für die Übertragung der Alkyl- oder des Benzyl-Restes.

Die in dieser Dissertation genannten Ergebnisse zeigen, dass sowohl Allyl- als auch Benzyl-Analoga von DMAPP als mögliche Alternativen für chemoenzymatische Friedel–Crafts-Alkylierungen zur Herstellung unnatürlich alkylierter Substanzen verwendet werden können.

1. Introduction

1.1. Ascomycota and Actinobacteria

Ascomycota comprise over 325 families in more than 60 orders and constitutes the largest phylum in the kingdom of fungi (Kirk *et al.*, 2008). Ascomycota are wide spread in many terrestrial and marine habitats (Manohar and Raghukumar, 2013) and live as saprophytes (Barata *et al.*, 2012), endophytes (Suryanarayanan, 2013) or plant- as well as animal-parasites (Ceryngier and Twardowska, 2013; Takamatsu, 2013). One type of growth is living as unicellular yeasts and reproducing by either asymmetric or symmetric division called budding or fission, respectively. Another type is spreading in multicellular hyphae and forming mycelia. This filamentous form in turn can propagate either asexually by development of conidia or sexually by development of spores enclosed in a sac like cell, the so-called ascus. For this reason, these fungi are termed ascomycetes (Webster and Weber, 2007).

Especially, the two genera *Aspergillus* and *Penicillium* have been of long standing interest. Different *Aspergillus* spp. are used for the industrial production of citric acid, itaconic acid (**1**), kojic acid (**2**), soy sauce and other far eastern food products (Aidoo *et al.*, 1994; Bentley, 2006; Karaffa and Kubicek, 2003; Nout and Aidoo, 2002; Steiger *et al.*, 2013). Mold cheeses are manufactured with the assistance of species from the genus *Penicillium*, e.g. *Penicillium roqueforti* (*P. roqueforti*) and *Penicillium camenberti* (Jakobsen *et al.*, 2002). Secreted enzymes, instead of the whole organisms, have been used extensively in industrial applications (Oxenbøll, 1994) and new sources of such enzymes, e.g. *Aspergillus parasiticus* for keratinolytic proteases or *Aspergillus versicolor* (*A. versicolor*) for cellulose degrading CMCases, were examined recently (Anitha and Palanivelu, 2013; Qaisar *et al.*, 2014). An important pharmaceutically used secondary metabolite is the cholesterol lowering agent lovastatin (**3**), which is produced mainly by *Aspergillus terreus* (*A. terreus*) (Alberts *et al.*, 1980). *Penicillium chrysogenum* is of great importance due to its ability to produce the antibiotic penicillin (G; **4**) and therefore researchers continuously strive to improve its production capabilities (Scheckhuber *et al.*, 2013; Wang *et al.*, 2014; Weber *et al.*, 2012). However, fungi of the abovementioned genera and secondary metabolites thereof can also threaten human health. In immunosuppressed patients, *Aspergillus fumigatus* (*A. fumigatus*) may cause the respiratory disease aspergillosis, which can afflict other inner organs including the central nervous system in severe cases (Denning, 1998; Desoubeaux *et al.*, 2014). Species of both, *Aspergillus* as well as *Penicillium*, are also considered as potential allergenic sources

(Crameri *et al.*, 2014). Secondary metabolites such as the mycotoxins aflatoxin B_1 (**5**) and ochratoxin A (**6**) from *Aspergillus* or *Penicillium spp.* are strong carcinogenic agents (el Khoury and Atoui, 2010; Kensler *et al.*, 2011).

Actinobacteria are one of the largest phyla in the bacterial kingdom. Members thereof are gram-positive bacteria with guanine and cytosine rich genomic DNA. Actinobacteria of the genus *Streptomyces* are indigenous to soil and aquatic environments (Pathom-aree *et al.*, 2006; Veyisoglu and Sahin, 2014) and are also found in a symbiotic relationship in higher plants (Tanvir *et al.*, 2014). Distinct from other bacteria, members of this genus grow in mycelia, form hyphae and sporulate similar to filamentous fungi (Chater, 1993; Flardh and Buttner, 2009).

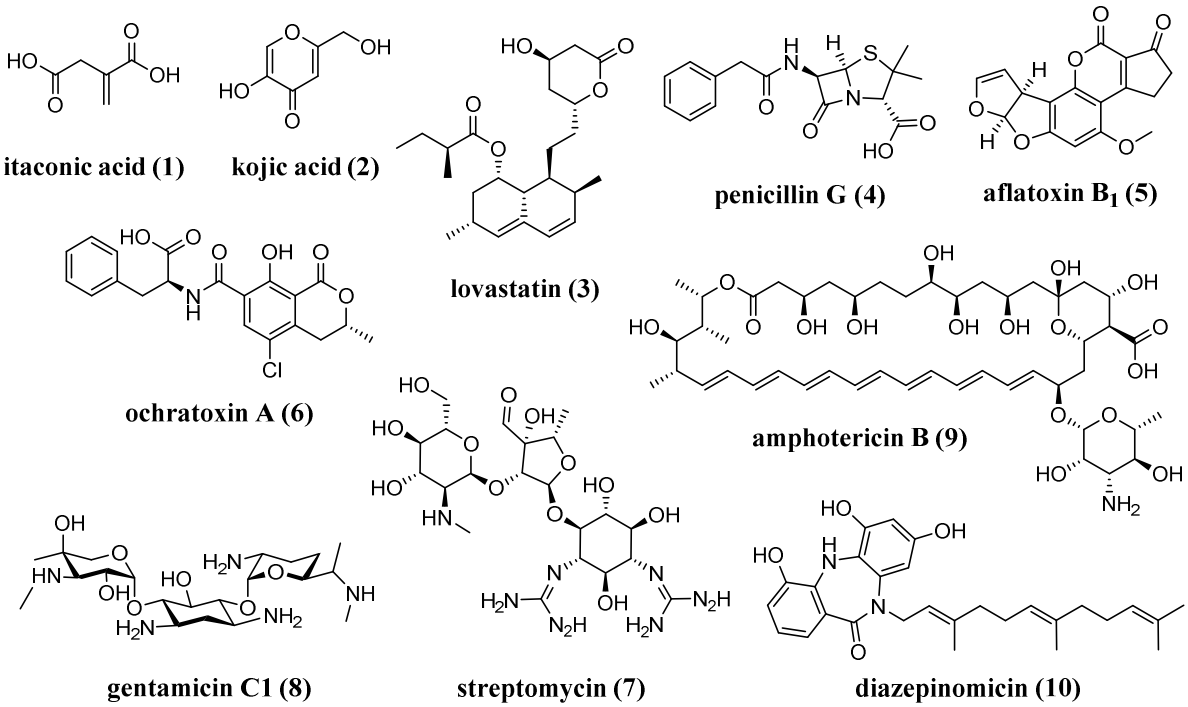


Figure 1.1 A selection of secondary metabolites from different *Aspergillus* spp., *Penicillium* spp. or *Streptomyces* spp..

Streptomycetes cover the production of over 75% of natural products from actinobacteria (Watve *et al.*, 2001). Since the discovery of the antibiotic streptomycin (**7**) in 1944 by Schatz *et al.* a multitude of pharmacologically active substances, e.g. gentamicin (C1; **8**), amphotericin B (**9**) and diazepinomycin (**10**), have been found in diverse *Streptomyces* spp. and other actinobacteria (Lemke *et al.*, 2005; Manivasagan *et al.*, 2014; Procopio *et al.*, 2012). Aside from antibiotic effects, anti-inflammatory (Ali *et al.*, 2013), anti-viral (Serkedjieva *et al.*, 2012) and anti-tumor (Lu *et al.*, 2012) activities have been observed. However, the rate of new bioactive compounds isolated from terrestrial streptomycetes diminished over the years due to the limited natural production of these compounds. New cryptic biosynthetic pathways were identified through the sequencing of the complete genomes of several *Streptomyces* spp., e.g.

Streptomyces coelicolor (*S. coelicolor*) A3(2), *Streptomyces avermitilis* and *Streptomyces griseus* IFO 13350 (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). Subsequent genome mining approaches helped to reveal several new compounds (Gomez-Escribano *et al.*, 2012; Laureti *et al.*, 2011). Marine actinobacteria including many *Streptomyces* spp. have also been of increasing interest as another source for new bioactive secondary metabolites (Manivasagan *et al.*, 2014).

1.2. Prenylated aromatic secondary metabolites

Prenylated aromatic secondary metabolites are distributed throughout all kingdoms of life. These compounds fulfill various roles in their plant, bacterial or fungal hosts and the prenyl residue is a key element for the presented biological and pharmacological activities (Botta *et al.*, 2005; El-Seedi *et al.*, 2010; Heide, 2009b; Li, 2010; Sunassee and Davies-Coleman, 2012). The basic chemical structures of these metabolites emerge from different primary biosynthesis pathways and form various substance classes such as indoles, flavonoids, coumarins, xanthones, quinones and naphthalenes. Onto these scaffolds, the naturally predefined prenyl moiety, e.g. dimethylallyl (DMA), is then attached.

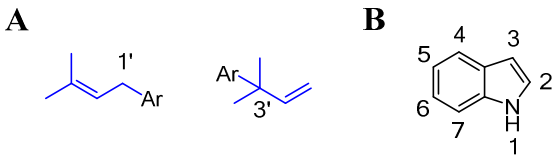


Figure 1.2 A: Orientation of the prenyl moiety using the example of DMAPP; B: numbering of the indole ring.

The prenyl residue can occur in two possible orientations after being transferred to the aromatic compound. For a regular prenylation, the allyl moiety is attached via C-1', whereas for a reverse prenylation, the allyl moiety is attached via C-3' (Figure 1.2; A) onto the aromatic ring (Ar). The prenylation position is labeled according to the numbering of the scaffold the residue is attached to. In case of an indole moiety, the positions are numbered N-1 and C-2 to C-7 (Figure 1.2; B).

1.2.1. Prenylated flavonoids

In plants, prenylated flavonoids are the largest group of prenylated secondary metabolites and production of these compounds was investigated intensively in the families of Fabaceae (Leguminosae) and Moraceae (Botta *et al.*, 2005). Other notable families are Euphorbiaceae (Zakaria *et al.*, 2012), Apiaceae (Umbelliferea) (Ohnogi *et al.*, 2012) and Cannabaceae (Wesolowska *et al.*, 2014; Yuan *et al.*, 2014). Flavonoids are polyphenolic compounds

including a 1,4-benzopyrone structure. Prenylated flavonoids exhibit anti-inflammatory properties (Hosek *et al.*, 2011), vasorelaxant (Kim *et al.*, 2013), antioxidative (Lan *et al.*, 2013), hepato-protective (Yang *et al.*, 2013), estrogenic (Simons *et al.*, 2012) and anti-*Staphylococcus aureus* activities (Manner *et al.*, 2013; Sasaki *et al.*, 2012). The majority of the identified prenylated flavonoids belong to the subclasses of flavones (Botta *et al.*, 2005), isoflavones (Botta *et al.*, 2009) and chalcones (Sasaki *et al.*, 2011).

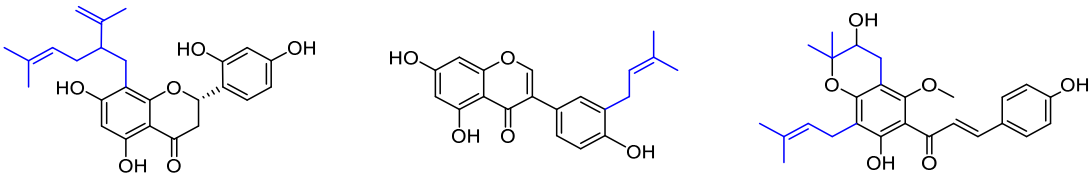


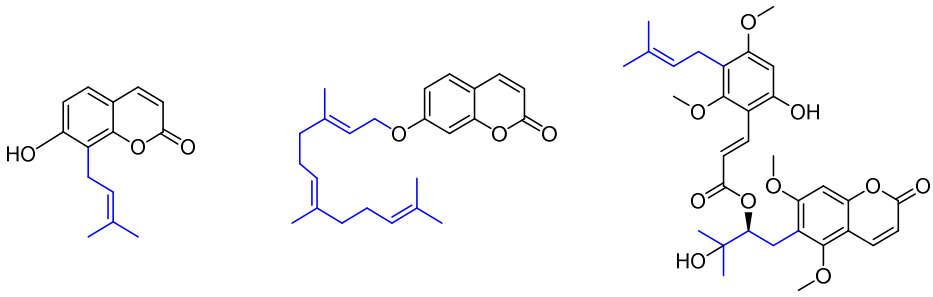
Figure 1.3 Examples of prenylated flavonoids.

The prenyl residue usually is attached to a carbon atom of the benzopyrone structure as in sophoraflavone G (**11**) (Yamamoto *et al.*, 1991) or glyceollins (Lyne *et al.*, 1976) obtained from *Sophora flavescens* or *Glycine max*, respectively. Further examples of C-prenylated flavonoids are the isoflavone isowighteone (**12**) from *Lupinus albus* (Shen *et al.*, 2012) and the chalcone xanthohumol M (**13**) from *Humulus lupulus* (Yu *et al.*, 2014). Apart from the aforementioned C-prenylation, O-prenylated flavonoids have also been identified, such as amyrisin A-C from *Amyris madrensis* (Peng *et al.*, 2012). Rare, but also found in nature is the combination of prenylated and at the same time glycosylated flavonoids (Koyama *et al.*, 2011).

1.2.2. Prenylated coumarins

Prenylated coumarins are produced mainly by plants, but also by bacteria as well as fungi and exhibit strong pharmacological activity (Heide, 2009b; Venugopala *et al.*, 2013). A lot of the prenylated coumarin-producing genera belong to the family of Apiaceae (Gliszczynska and Brodelius, 2012). The isoprenoid moiety is affixed to a carbon or oxygen of the hydroxylated 1,2-benzopyrone framework. Plants of the species *Artemisia* and *Fatoua* provide mono- and di-prenyl coumarins, respectively (Chiang *et al.*, 2010; Mojarrab *et al.*, 2011). In parsley linear and angular furanocoumarins have been identified, which are derived from 6-DMA-umbelliferone (demethylsuberosin) and 8-DMA-umbelliferone (osthenol; **14**), respectively (Karamat *et al.*, 2014). Organic extracts of coumarin-containing plants as well as isolated prenylated coumarins such as umbelliprenin (**15**) demonstrated antifungal activity (Kurdelas *et al.*, 2010), apoptotic effects on cancer cells (Gholami *et al.*, 2013; Haghighi *et al.*, 2014) or anti-inflammatory effects (Huang *et al.*, 2012). The anti-phosphodiesterase-4 activity

of prenylated coumarins such as toddalin B (**16**) is of interest for the treatment of pulmonary diseases (Lin *et al.*, 2014).

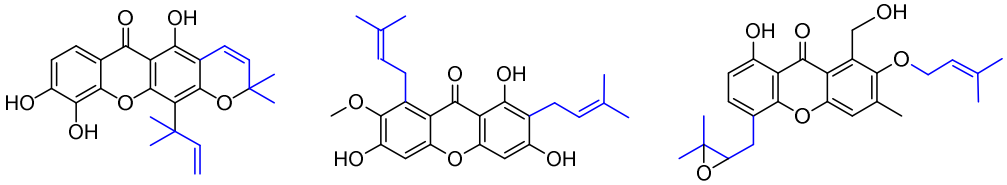


osthenol (14) **umbelliprenin (15)** **toddalin B (16)**

Figure 1.4 Examples of prenylated coumarins.

1.2.3. Prenylated xanthones

Plants, fungi and lichen are producers of xanthones and derivatives thereof (El-Seedi *et al.*, 2009; Masters and Bräse, 2012; Vieira and Kijjoa, 2005). Numerous investigations concentrated on prenylated xanthones from *Garcinia* sp. (Tantapakul *et al.*, 2012; Xia *et al.*, 2012; Xu *et al.*, 2014). The prenyl moieties are attached usually at the carbons of the 9*H*-xanthen-9-one nucleus in ortho-position of a hydroxyl group. Butyraxanthone F (Tala *et al.*, 2013) and macluraxanthone (**17**) (Teh *et al.*, 2013) found in *Pentadesma butyracea* and *Mesua* spp., respectively, are examples of C-prenylated xanthones. Oblongifolixanthone A is a three times prenylated xanthone isolated from *Garcinia oblongifolia*, which exhibits moderate cytotoxic effects. Promising anti-cancer effects (Aisha *et al.*, 2012), antimicrobial properties (Al-Massarani *et al.*, 2013) and antihyperglycemic activity of xanthone containing extracts, including α-mangostin (**18**), were also reported recently (Ryu *et al.*, 2011).



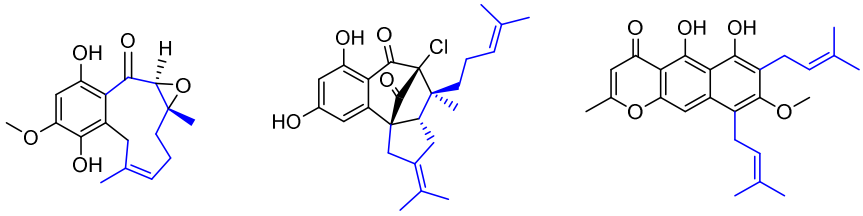
macluraxanthone (17) **α-mangostin (18)** **variecoxanthone C (19)**

Figure 1.5 Examples of prenylated xanthones.

The genera of *Aspergillus* and *Penicillium* are fungal producers of prenylated xanthones (Masters and Bräse, 2012; Sanchez *et al.*, 2011; Simpson, 2012). From these fungi, e.g. *Aspergillus nidulans* (*A. nidulans*), C- and O-prenylated xanthones such as shamixanthone and variecoxanthone C (**19**) have been identified (Masters and Bräse, 2012; Simpson, 2012).

1.2.4. Prenylated quinones and naphthalenes

Prenylated quinones, hydroxyquinones and naphthoquinones belong to the group of meroterpenoids, which are hybrid natural products consisting of metabolites of the polyketide as well as the terpene biosynthetic pathway. These secondary metabolites are widespread natural products and have been isolated from diverse marine organisms such as bacteria, fungi, algae and sponges (Sunassee and Davies-Coleman, 2012; Suzuki *et al.*, 2014) as well as terrestrial organisms such as plants, fungi and bacteria (Gale *et al.*, 1963; Madrid *et al.*, 2012; Sedmera *et al.*, 1991).



terreumol C (20) **merochlorin A (21)** **adenaflorin B (22)**

Figure 1.6 Examples of prenylated quinones and naphthalenes.

From the alga *Perithalia capillaris*, for example, regular and reverse di-prenylated quinones (Sansom et al., 2007) and from the fungus *Tricholoma terreum* highly oxygenated cyclic geranyl-hydroxyquinones, e.g. terreumol C (**20**), were obtained (Yin et al., 2013b). The potential usage of meroterpenoids as anti-inflammatory (de los Reyes et al., 2013) or insecticidal (Centko et al., 2014) agents has been investigated in recent years.

Prenylated naphthalenes, on the other hand, are not very abundant in nature. Naturally occurring compounds are the antibiotic merochlorin A (**21**) from *Streptomyces* sp. CNH-189 (Kaysser et al., 2012) as well as the cytotoxic adenaflorin A-D (B; **22**) isolated from *Adenaria floribunda* (Hussein et al., 2004).

1.2.5. Prenylated indole alkaloids

Indole alkaloids comprised of an indole or indole-containing backbone and an isoprene modification are naturally occurring secondary metabolites (Li, 2010). The indole structure is derived from L-tryptophan and derivatives thereof, e.g. linear dipeptides, cyclic dipeptides or cyclic tripeptides biosynthesized by non-ribosomal peptide synthases (NRPS) (Haynes *et al.*, 2013; Kalb *et al.*, 2013; Marahiel, 2009). In some cases, prenylated indoles are derived from a precursor in the tryptophan biosynthesis, e.g. indole diterpenoids (Cai *et al.*, 2013; Saikia *et al.*, 2008). The principal producers are fungi of the genera *Aspergillus* and *Penicillium* (Finefield *et al.*, 2012; Li, 2010; Ruiz-Sanchis *et al.*, 2011), but prenylated compounds can also be found in bacteria or plants (Haque *et al.*, 2013; Raju *et al.*, 2011). They carry prenyl moieties on

various positions of the indole ring (Li, 2010). These natural products attracted attention in consequence to their strong biological and pharmacological effects (Li, 2010; Wallwey and Li, 2011). Therefore, diverse approaches are used to obtain such compounds, for example isolation from novel natural sources (Peng *et al.*, 2013), chemical synthesis (Simpkins *et al.*, 2013), chemoenzymatic synthesis (Li, 2010), and with the help of interdisciplinary approaches (Sunderhaus *et al.*, 2013a).

This thesis deals with indole prenyltransferases that catalyze the formation of these compounds from L-tryptophan or tryptophan-containing cyclic dipeptides. Thus, prenylated indole alkaloids containing these structures are described below in more detail.

1.2.5.1. Prenylated indole alkaloids derived from L-tryptophan

The most prominent representatives of this group are ergot alkaloids, which are found primarily in the genera *Aspergillus* and *Claviceps* (Schardl *et al.*, 2006; Wallwey and Li, 2011). The first common precursor of these alkaloids is 4-DMA-L-tryptophan. Ergot alkaloids and their semisynthetic derivatives are well known for their strong toxic effects (Schardl *et al.*, 2006) as well as their pharmaceutical utility including migraine prevention (Haarmann *et al.*, 2009) postpartum hemorrhage prevention (Ezeama *et al.*, 2014) and treatment of Parkinson's as well as Alzheimer's disease (Bonuccelli *et al.*, 2009; Winblad *et al.*, 2008). In diverse *Penicillium* spp., the secondary metabolites rugulovasine A, B and the immunotoxic α-cyclopiazonic acid (**23**) have been isolated, which have the C-4 attached prenyl moiety incorporated into a multi-ring structure similar to that of ergot alkaloids (Abe *et al.*, 1969; Holzapfel *et al.*, 1970; Hymery *et al.*, 2014). The tremorgenic toxin aflatrem is a C4-reverse prenylated indole diterpene found in *A. flavus* (Gallagher and Wilson, 1978).

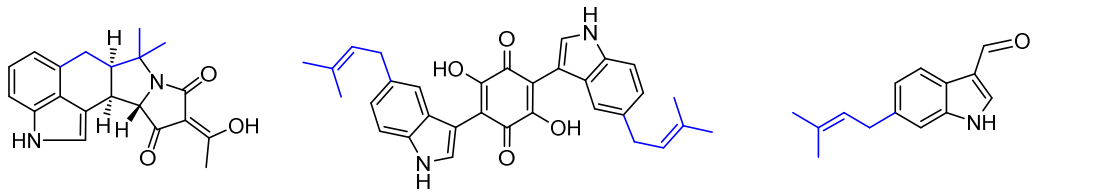


Figure 1.7 Examples of prenylated indole alkaloids.

Examples of C5-prenylated indole alkaloids are shearinine D and E from *Penicillium janthinellum* (Smetanina *et al.*, 2007) or the bis-indolyl-benzoquinones semicochliodinol A and cochliodinol (**24**) from *Chrysosporium mevdarium* and *Chaetomium* sp. (Brewer *et al.*, 1968; Fredenhagen *et al.*, 1997). For the latter two compounds, antimicrobial and cytotoxic effects have been reported (Casella *et al.*, 2013). Semicochliodinol B and isocochliodinol are the respective C6-prenylated counterparts of semicochliodinol A and cochliodinol (Fredenhagen *et*

al., 1997). The natural metabolite 6-DMA-indole-3-carbaldehyde (**25**) was identified with the help of biochemical investigations of the prenyltransferase IptA from *Streptomyces* sp. SN-593 (Takahashi *et al.*, 2010). Indole derivatives with prenyl residues at position C-6 such as annonidine F and caulidine A, obtained from African medical plants, have shown anti-malarial activity (Onguene *et al.*, 2013).

1.2.5.2. Prenylated indole alkaloids derived from tryptophan-containing dipeptides

The cyclic dipeptides consist either of two L-tryptophan units or one L-tryptophan and another aromatic or aliphatic amino acid (Li, 2010). The majority of the prenylated cyclic dipeptides is derived from *cyclo*-L-tryptophanyl-L-prolinyl, i.e. brevianamide F (Williams *et al.*, 2000). Fumitremorgins are a group of C2-DMA-brevianamide F (tryprostatin B; Scheme 1.1) derivatives and are mycotoxins that exhibit strong tremorgenic effects, anti-cancer activity (Borthwick, 2012; Frisvad *et al.*, 2009) and also showed antifungal properties (Li *et al.*, 2012). These compounds, such as fumitremorgin A, verruculogen and several derivatives thereof, have been found in different *Aspergillus* and *Penicillium* strains (An *et al.*, 2014; Gallagher and Latch, 1977; Wang *et al.*, 2012; Yamazaki *et al.*, 1980). In addition, brevianamide F derived prenylated dipeptides such as notoamides (Chen *et al.*, 2013; Kato *et al.*, 2007; Tsukamoto *et al.*, 2010), stephacidins (Greshock *et al.*, 2008) and related substances (Peng *et al.*, 2013) have been isolated from different *Aspergillus* spp.. Compounds such as notoamide A (**26**) carry a reverse C2- or C3-prenylation, which is often incorporated into a bicyclo (2,2,2)-diazaoctane ring, as well as a modified regular C7-prenylation (Tsukamoto *et al.*, 2008; Tsukamoto *et al.*, 2010).

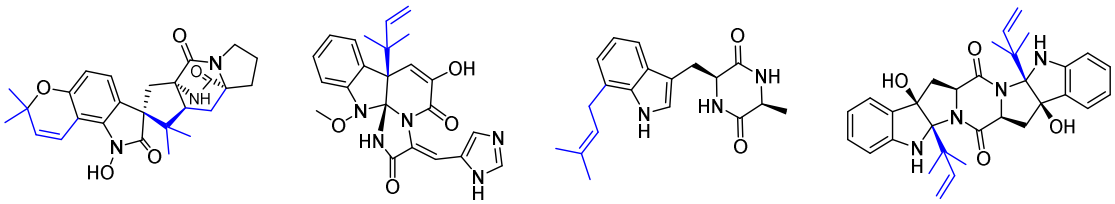


Figure 1.8 Examples of prenylated indole dipeptides.

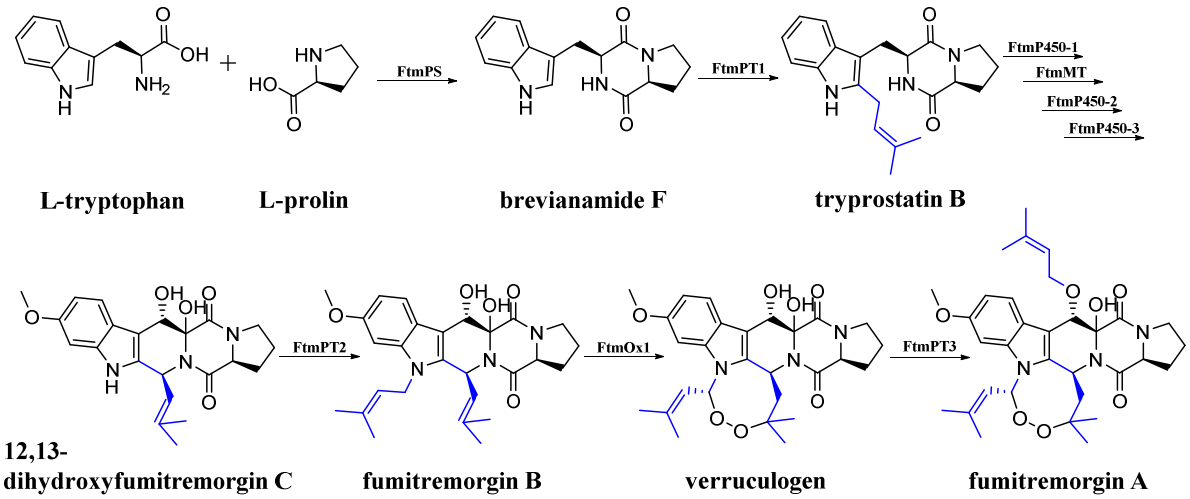
In the case of *cyclo*-L-tryptophanyl-L-histidinyl, another suitable prenylation substrate, the prenyl moiety is placed usually reversely at C-3 of the indole ring. Examples of such compounds are the prenylated secondary metabolite roquefortine C, obtained from *P. roqueforti* (Scott and Kennedy, 1976) and its more complex derivative meleagrin (**27**), isolated from *Penicillium glandicola* (Reshetilova *et al.*, 1995). Meleagrine and derivatives thereof have shown antibacterial potential as FabI inhibitors (Zheng *et al.*, 2013). Further examples of prenylated cyclic dipeptides are terezine D (**28**) from *Sporormiella teretispora*, which is a

C7-DMA-L-tryptophanyl-L-alaninyl cyclic dipeptide (Wang *et al.*, 1995) or the C2-prenylated fellutanine D (**29**), consisting of two L-tryptophanyl moieties, isolated from *Penicillium fellutanum* (Kozlovsky *et al.*, 2000; Kozlovsky *et al.*, 2001).

1.3. Biosynthesis of prenylated aromatic secondary metabolites

For better understanding of the assembling mechanisms of prenylated indole alkaloids, several gene clusters responsible for their biosynthesis have been studied intensively in recent years. For this purpose, diverse methods such as genome mining, feeding and gene deletion experiments as well as biochemical characterization have been applied. Our research group focused on the biosynthetic pathways of fumitremorgins and fumigaclavines with special emphasis on the involved prenyltransferases. Moreover, some of these prenyltransferases were used in diverse experiments performed in this thesis. Therefore, both pathways are described in detail in the following paragraphs.

Scheme 1.1 Biosynthetic pathway of fumitremorgin A in *N. fischeri* (modified from Mundt *et al.*, 2012).



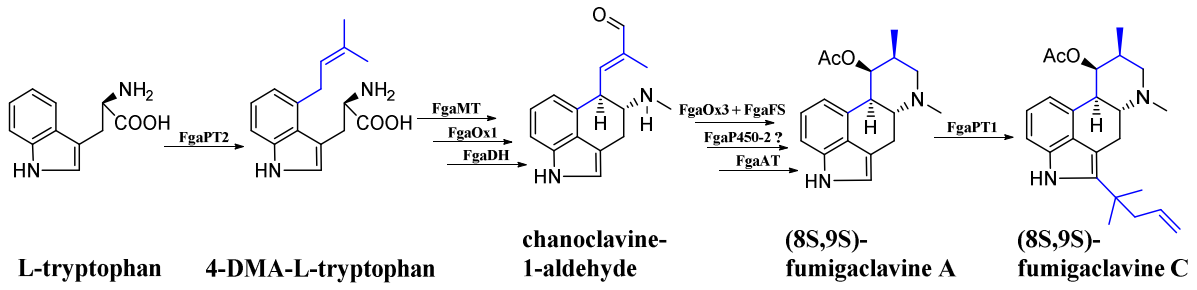
The biosynthetic pathway of the fumitremorgins is found in *A. fumigatus* and *Neosartorya fischeri* (*N. fischeri*) (Scheme 1.1). The next intermediate, following the assembly of L-tryptophan and L-prolin to brevianamide F, is the C2-prenylated cyclic dipeptide tryprostatin B catalyzed by FtmPT1 (Grundmann and Li, 2005; Maiya *et al.*, 2006). After multiple modification steps catalyzed by P_{450} enzymes and a methyltransferase (Kato *et al.*, 2009), the intermediate 12,13-dihydroxyfumitremorgin C is converted to fumitremorgin B by subsequent N-prenylation with FtmPT2, followed by an endoperoxidation to verruculogen with FtmOx1. Verruculogen is the end-product of the biosynthetic pathway in *A. fumigatus* (Grundmann *et al.*, 2008; Steffan *et al.*, 2009). The final step of this biosynthetic pathway of *N. fischeri* is the O-prenylation of verruculogen to fumitremorgin A by FtmPT3. These biochemical

investigations have also shown, that not all involved genes have to be clustered and that they can be found on different chromosomes of the same organism (Mundt *et al.*, 2012).

The gene cluster for biosynthesis of notoamides and stephacidins also uses *cyclo*-L-tryptophanyl-L-prolinyl as a starter molecule and its central element is the C2-reverse prenylated deoxybrevianamide E (Ding *et al.*, 2010). Investigations on both pathways included feeding experiments with chemically synthesized isotopic intermediates (Finefield *et al.*, 2011a; Finefield *et al.*, 2011b; Sunderhaus *et al.*, 2013b).

The other aforementioned biosynthetic pathway, studied in our research group, is that of the clavine-type ergot alkaloids. The enzyme FgaPT2 catalyzes the C4-prenylation of L-tryptophan, the first pathway-specific step for fumigaclavines in *A. fumigatus* (Scheme 1.2) (Unsöld and Li, 2005). Subsequent methylation, oxidation and dehydrogenation of 4-DMA-L-tryptophan lead to chanoclavine-1-aldehyde (Coyle *et al.*, 2010; Rigbers and Li, 2008; Wallwey *et al.*, 2010a). These reactions are followed by a cyclization reaction catalyzed by the two enzymes FgaOx3 and FgaFS (Wallwey *et al.*, 2010b) and two further steps including a hydroxylation and an acetylation reaction leading to the last intermediate (8S,9S)-fumigaclavine A (Liu *et al.*, 2009; Wallwey and Li, 2011). The enzyme FgaPT1 is responsible for the final reaction step in the clavine biosynthesis, catalyzing the C2-prenylation of (8S,9S)-fumigaclavine A to (8S,9S)-fumigaclavine C (Unsöld and Li, 2006).

Scheme 1.2 Biosynthetic pathway of (8S,9S)-fumigaclavine C in *A. fumigatus* (modified from Wallwey and Li, 2011).



The prenylation reaction catalyzed by DmaW, a homologue of FgaPT2, is an essential step in the biosynthesis of ergot alkaloids derived from D-lysergic acid in *Claviceps purpurea* (*C. purpurea*) (Flieger *et al.*, 1997; Tsai *et al.*, 1995). Up to the formation of the branch point intermediate chanoclavine-1-aldehyde, the clavine type as well as the D-lysergic acid type ergot alkaloid pathways share the same enzymatic reaction steps. Further reactions lead to D-lysergic acid and finally ergoamides such as ergometrine or ergopeptines such as ergotamine (Wallwey and Li, 2011).

A rather short biosynthetic pathway is the acetylaszonalenin pathway, which consists only of three genes and uses L-tryptophan and anthranilic acid as starter molecules. The resulting cyclic dipeptide is reversely prenylated at C-3 of the indole ring prior to an acetylation reaction (Yin *et al.*, 2009b). Apart from biosynthetic pathways of prenylated tryptophan-containing dipeptides, those with tripeptidyl origin have also been investigated (Haynes *et al.*, 2013).

1.4. Prenyltransferases

Prenyltransferases encompass a wide family of enzymes found in all living organisms, creating substances of the primary as well as secondary metabolism. These enzymes catalyze the cleavage of a prenyl moiety from its diphosphate and the subsequent transfer of the resulting isoprene carbocation onto other isoprenoid moieties, amino acids or aromatic structures as well as a few non-aromatic structures (Heide, 2009a). The prenyl donors, i.e. prenyl diphosphates, originate from the terpenoid biosynthesis pathway and therefore consist of n × C-5 units, e.g. dimethylallyl diphosphate (DMAPP; n=1), geranyl diphosphate (GPP; n=2), farnesyl diphosphate (FPP; n=3) or geranylgeranyl diphosphate (GGPP; n=4) (Heide, 2009a).

1.4.1. *Cis*- and *trans*-prenyltransferases

FPP synthases are part of the class of short chain *trans*-prenyltransferases and catalyze the assembly of all-*trans* configured FPP that serves as a key element in the formation of a multitude of isoprenoids (Dhar *et al.*, 2013). Plant secondary metabolites like carotenoids, in turn, are formed by GGPP synthases (Liu *et al.*, 2014). Octaprenyl diphosphate synthases (8 × C-5 units) belong to an enzyme group that catalyze medium to long (6-10 × C-5 units) chain elongations of *trans*-prenyl diphosphates. These prenyl residues can then be attached to different quinone structures leading to substances such as ubiquinone or menaquinone (Soballe and Poole, 1999).

In contrast, *cis*-prenyltransferases condense even longer *Z/E*-mixed isoprenoid chains. Undecaprenyl diphosphate consists of 11 × C5 units and is used as lipid carrier in the peptidoglycan biosynthesis of bacteria (Teng and Liang, 2012). Dolichols usually range from 15 to 20 isoprene units and are for example important for protein glycosylation and cell wall stability in *Saccharomyces cervisiae* (*Sa. cerevisiae*) and *Candida albicans* (*C. albicans*) (Juchimiuk *et al.*, 2014; Orlowski *et al.*, 2007). All these substances are dwarfed by industrially used natural rubbers, which exceed average molecular weights of 1,000 kDa and therefore comprise over 15,000 isoprene units, biosynthesized by cis-prenyltransferases from plants such as *Taraxacum* spp. (Post *et al.*, 2012; van Beilen and Poirier, 2007).

1.4.2. Protein prenyltransferases

Another important subclass of prenyltransferases are protein prenyltransferases. The common prenyl donors are FPP or GGPP and the resultant carbocations are attached to an activated cysteine in a CAAX motif of the corresponding protein and serve as posttranslational modifications (Moores *et al.*, 1991; Zhang and Casey, 1996). Due to the involvement of farnesylated proteins in oncogenic reactions, the underlying prenylation reaction and its inhibition have been research subjects for many years (Abuhaie *et al.*, 2013; Pham *et al.*, 2006; Sousa *et al.*, 2009; Zhu *et al.*, 2014).

1.4.3. Aromatic prenyltransferases

Aromatic prenyltransferases are deeply involved in the biosynthesis of many secondary metabolites. These enzymes catalyze the transfer reaction of a prenyl residue onto structures such as phenols, phenolic acids, naphthalenes, flavonoids and indoles (Botta *et al.*, 2005; El-Seedi *et al.*, 2009; Heide, 2009b; Li, 2010). The acceptance of aromatic compounds by these enzymes is one explicit feature to divide the diverse prenyltransferases into distinct enzyme groups. Further discrimination characteristics are their native organism and if the proteins are soluble in the cytosol or membrane bound. Dependency on divalent metal ions is an additional criterion. Moreover, aromatic prenyltransferases may present certain amino acid motifs or exhibit distinct tertiary structures. There are several possibilities to categorize prenyltransferases based on the described properties. One classification variant of the prenyltransferases comprises the UbiA superfamily, the CloQ/NphB group, the LtxC group and the DMATS superfamily. These groups or superfamilies are described in the following paragraphs (Heide, 2009a).

1.4.3.1. Prenyltransferases of the UbiA superfamily

Enzymes of this family are termed after the prenyltransferase UbiA, responsible for an essential step in the biosynthesis of ubiquinones. The original reaction catalyzes the substitution of an all-*trans* octaprenyl moiety onto 4-hydroxybenzoic acid by UbiA and COQ2 from *Escherichia coli (E. coli)* and *Sa. cerevisiae*, respectively (Ashby *et al.*, 1992; Melzer and Heide, 1994). Members of this family are also involved in the biochemical synthesis of menaquinones, plastoquinones, heme groups and vitamin E (Cheng and Li, 2014). The enzymes of this class carry an aspartate rich NXxxDxxxD motif and their reactions are dependent on divalent metal ions such as Mg^{2+} and Mn^{2+} as mediators in the catalytic dissociation of diphosphate from the polyprenyl moiety (Melzer and Heide, 1994; Stec and Li, 2012). Furthermore, these

prenyltransferases are membrane bound due to respective anchoring domains found in their amino acid sequence (Cheng and Li, 2014; Melzer and Heide, 1994).

LePGT-1 and LePGT-2 are two isoenzymes from *Lithospermum erythrorhizon* and are involved in the geranylation reaction of shikonin (Ohara *et al.*, 2009; Yazaki *et al.*, 2002). Further members of this superfamily catalyze the prenylation of naringenin, gestigenin, isoliquiritigenin as well as glyceollin in *Sophora flavescens* as well as *Glycine max*, respectively (Akashi *et al.*, 2009; Sasaki *et al.*, 2008; Sasaki *et al.*, 2011). Another entry into this enzyme class is AuaA, which is involved in the biosynthesis of aurachin D in the myxobacterium *Stigmatella aurantiaca* (Stec *et al.*, 2011). The UbiA homologue UBIAD1 was discovered in human cells and is involved in the vitamin K biosynthesis, which is important for maintaining vascular homeostasis (Hegarty *et al.*, 2013; Nakagawa *et al.*, 2010). Recently, the crystallization of UbiA revealed a tertiary structure consisting of nine alpha helices embedded in the membrane and three helices that form a cap facing the cytosol. The entrance to the central cavity is facing sideways into the membrane, enabling the prenylation of quinones using long prenyl diphosphates and the direct release of the products into the membrane. These findings gave detailed information about the reaction mechanism of this type of prenyltransferases (Cheng and Li, 2014).

1.4.3.2. Prenyltransferases of the CloQ/NphB group

The enzyme CloQ from *Streptomyces roseochromogenes* catalyzes the prenylation of 4-hydroxyphenylpyruvic acid in the clorobiocin biosynthesis (Pojer *et al.*, 2003). NphB from *Steptomyces* sp. CL190 is involved in the biochemical synthesis of naphterpin (Kuzuyama *et al.*, 2005). In contrast to the membrane bound enzymes of the UbiA superfamily, this group comprises soluble enzymes found in bacteria as well as fungi (Heide, 2009a). While these enzymes also catalyze the prenylation of phenolic compounds, no aspartate rich (N/D)DxxD motif is present and with the exception of NphB, all enzymes of this group do not depend on divalent metal ions (Kuzuyama *et al.*, 2005; Tello *et al.*, 2008). Crystallization of NphB revealed a new tertiary structure with an α-β-β-α (ABBA) barrel fold (Figure 1.9). This protein structure is also termed prenyltransferase (PT) barrel, which allows another classification into the ABBA prenyltransferase family (Kuzuyama *et al.*, 2005; Tello *et al.*, 2008). This PT-barrel is distinct from the triose-phosphate isomerase barrel, i.e. TIM-barrel, which features an eight times recurring β-α motif (Figure 1.9) (Banner *et al.*, 1975; Wierenga *et al.*, 2010). Recent computational studies further elucidated and strengthened the metal ion independent prenylation mechanism of these enzymes (Bayse and Merz, 2014).

Furthermore SCO7190, an NphB homologue found in *S. coelicolor* A3(2), catalyzes the attachment of DMAPP onto 1,6-dihydroxynaphthalene (Kuzuyama *et al.*, 2005). PpzP belongs to the biosynthesis cluster responsible for prenylated phenazine-1-carboxylic acid in *Streptomyces anulatus* 9663 (Saleh *et al.,* 2009). In 2012, Zocher *et al.* discovered the second phenazine prenyltransferase EpzP in *S. cinnamonensis* DSM 1042 and elucidated its crystal structure. These enzymes are restricted to the transfer of C-5 or C-10 isoprene units. The first farnesyl diphosphate prenyltransferase of the CloQ/NphB group, DzmP, has been characterized from the bacterium *Micromonospora sp*. RV115 by Bonitz *et al.* in 2013 and is involved in the biosynthesis of the benzodiazepine diazepinomicin. The newest entry into this enzyme group is the 1,6-dihydroxyphenazine prenyltransferase Mpz10 from a marine *Streptomyces* sp., which is the first membrane bound prenyltransferase using a phenazine derivative as substrate (Zeyhle *et al.,* 2014).

1.4.3.3. Bacterial prenyltransferases of the LtxC group

Members of the LtxC group prenylate indole-containing substrates. These enzymes carry no aspartate rich motif and are divalent metal ion independent (Edwards and Gerwick, 2004). They are predominantly expressed in bacterial hosts. Due to their common features and relatively high sequence similarity with fungal DMAT synthases, this group of prenyltransferases could likely be integrated into the DMATS superfamily.

LtxC from *Lyngbya majuscula* is a member of the lyngbyatoxin A–C biosynthetic pathway and geranylates the precursor (–)-indolactam V at position C-7 to lyngbyatoxin A (Edwards and Gerwick, 2004). More recent investigations on the biosynthetic cluster of teleocidin B from *Streptomyces blastmyceticus* led to the discovery of LtxC homologous prenyltransferase TleC (Awakawa *et al.,* 2014).

In the actinobacterium *Salinispora arenicola,* the reverse L-tryptophan N-prenyltransferase CymD is involved in the biosynthesis of the anti-inflammatory and anti-bacterial cyclic peptides cyclomarin and cyclomarazine, respectively (Schultz *et al.,* 2008; Schultz *et al.,* 2010). MpnD from *Marinactinospora thermotolerans* catalyzes the reverse attachment of DMA on C-7 of an indolactam scaffold (Ma *et al.,* 2012). IptA is the first biochemically characterized indole prenyltransferase from *Streptomyces sp*. (SN-593) that produces diverse C6-DMA indole derivatives and can likely be placed into the LtxC group despite relatively low sequence homology (Takahashi 2010). Biochemical investigations on prenyltransferases from *Streptomyces ambofaciens* (*S. ambofaciens*) and *Streptomyces violaceusniger* (*S. violaceusniger*) revealed two additional 6-DMAT synthases, namely 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$, respectively. (Winkelblech and Li, 2014).

1.4.3.4. Fungal prenyltransferases of the DMATS superfamily

Prenyltransferases of the **d**i**m**ethyl**a**llyl**t**ryptophan **s**ynthase (DMATS) superfamily are found predominantly in fungi of the genera of *Aspergillus*, *Penicillium* and *Claviceps* (Williams *et al.*, 2000). The enzymes are soluble proteins and carry no aspartate rich motifs. They catalyze mainly the formation of prenylated indole derivatives and no divalent metal ions are required for the catalytic reaction (Yu and Li, 2012). Most of these enzymes show broad substrate promiscuity while retaining a strong specificity regarding the prenylation position.

Table 1.1 A selection of indole prenyltransferases (PTs) from fungi and bacteria.

	strain	position	orientation	reference
L-tryptophan PTs				
FgaPT1	*A. fumigatus*	C-2	reverse	Unsöld and Li, 2006
FgaPT2	*A. fumigatus*	C-4	regular	Unsöld and Li, 2005
5-DMATS	*A. clavatus*	C-5	regular	Yu *et al.*, 2012b
SCO7467	*S. coelicolor*	C-5	regular	Ozaki *et al.*, 2013
IptA	*Streptomyces sp.* SN-593	C-6	regular	Takahashi *et al.*, 2010
6-DMATS$_{Sa}$	*S. ambofaciens*	C-6	regular	Winkelblech and Li, 2014
7-DMATS	*A. fumigatus*	C-7	regular	Kremer *et al.*, 2007
cyclic dipeptide PTs				
FtmPT1	*A. fumigatus*	C-2	regular	Grundmann and Li, 2005
CdpC2PT	*N. fischeri*	C-2	reverse	Mundt and Li, 2013
CdpC3PT	*N. fischeri*	C-3	reverse	Yin *et al.*, 2010
CdpC7PT	*A. terreus*	C-7	regular	Wunsch *et al.*, 2014
CTrpPT	*A. oryzae*	C-7/N-1	regular/reverse	Zou *et al.*, 2010
FtmPT2	*A. fumigatus*	N	regular	Grundmann *et al.*, 2008
FtmPT3	*N. fischeri*	O	regular	Mundt *et al.*, 2012
L-tyrosine PTs				
SirD	*L. maculans*	O	regular	Zou *et al.*, 2011
TyrPT	*A. niger*	O	regular	Fan *et al.*, 2014
other aromatic PTs				
AstPT	*A. terreus*	C-2/N-1	regular/reverse	Tarcz *et al.*, 2014a
TdiB	*A. nidulans*	C-2	reverse	Schneider *et al.*, 2008
ArdB	*A. fischeri*	C-3	reverse	Haynes *et al.*, 2013
VrtC	*P. aethiopicum*	C	regular	Chooi *et al.*, 2010
NscD	*N. fischeri*	C	regular	Chooi *et al.*, 2013
Mpz10	*Streptomyces* sp. SpC080624SC-11	C	regular	Zeyhle *et al.*, 2014
DmzP	*Micromonospora* sp. RV115	N	regular	Bonitz *et al.*, 2013
XptB	*A. nidulans*	O	regular	Pockrandt *et al.*, 2012

The first DMATS, 4-DMATS, was obtained from *C. purpurea* in 1992 by Gebler and Poulter and catalyzes the C4-prenylation of L-tryptophan. Later the corresponding gene *dmaW* was isolated, its amino acid sequence elucidated and the prenyltransfer reaction was proved in yeast transformants, therefore 4-DMATS is also termed DmaW (Tsai *et al.*, 1995). Prenyltransferases that attach the isoprene residue selectively to one of the carbon atoms (C-2, C-3, C-4, C-5, C-6, C-7) or the nitrogen atom (N-1) of the indole ring, in regular or reverse orientation, have been reported in recent years (Li, 2010; Yu and Li, 2012). Examples of fungal prenyltransferases of the DMATS superfamily together with bacterial prenyltransferases of the CloQ/NphB and LtxC group are summarized as L-tryptophan-, tryptophan-containing cyclic dipeptide-, L-tyrosine- and other aromatic substrate accepting prenyltransferases in the Table 1.1.

The enzymes categorized as aromatic PTs exhibit distinct features compared to the previously listed prenyltransferases regarding the substrates or donors used in the prenylation reaction. The substrates for AstPT as well as TdiB and ArdB, are a bisindolyl benzoquinone and a cyclic tripeptide, respectively (Haynes *et al.*, 2013; Schneider *et al.*, 2008; Tarcz *et al.*, 2014a). XptB combines xanthones with a DMA residue (Pockrandt *et al.*, 2012). VrtC was the first DMATS to utilize geranylgeranyl diphosphate (GPP) instead of DMAPP and moreover transferred the prenyl moiety onto a tetracyclic ring system (Chooi *et al.*, 2010). In addition, genome mining revealed a group of DMAPP transferring homologues to VrtC from different fungi, which accepted also tetracyclic naphthacenedione and in the case of NscD tricyclic anthracenones (Chooi *et al.*, 2012; Chooi *et al.*, 2013).

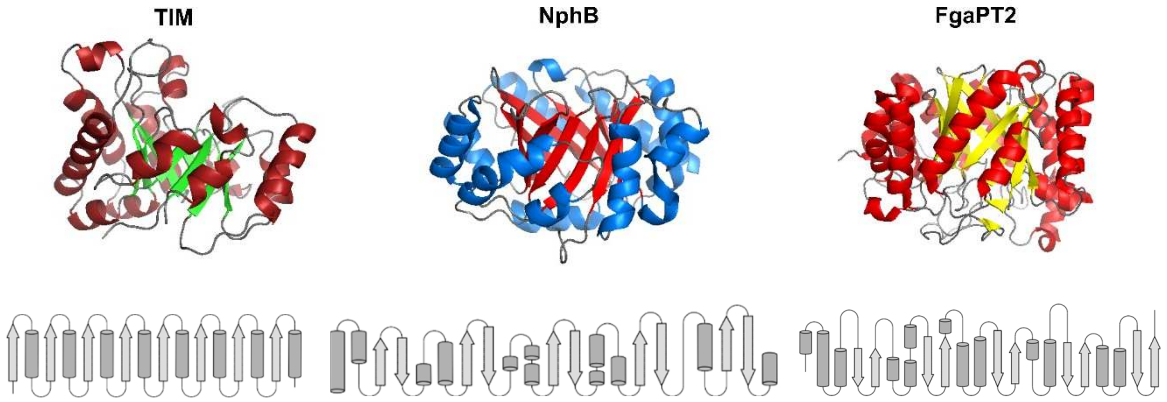


Figure 1.9 Top: side view of the tertiary structures of triose phosphate isomerase (PDB 5TIM) as well as NphB (formerly named Orf2; PDB 1ZB6) and FgaPT2 (PDB 3I4X) in cartoon presentation; Bottom: schematic illustrations of secondary structures of TIM, NphB and FgaPT2 (modified from Kuzuyama *et al.*, 2005; Metzger *et al.*, 2009; Wierenga *et al.*, 2010).

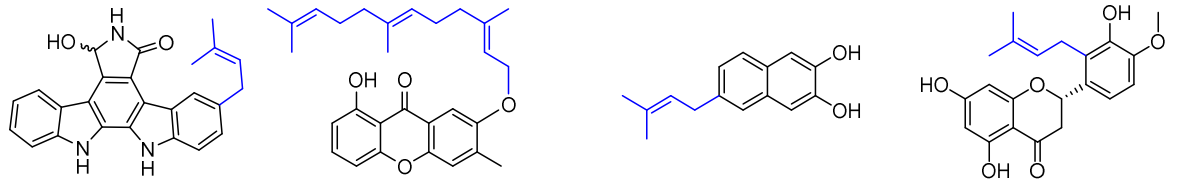
Structural analyses on the four indole prenyltransferases FgaPT2 (Figure 1.9) (Metzger *et al.*, 2009), FtmPT1 (Jost *et al.*, 2010), CdpNPT (Schuller *et al.*, 2012) and AnaPT (Yu *et al.*, 2013) provided new insights into the tertiary structure of DMAT synthases. All these fungal

prenyltransferases exhibit the ABBA fold, which has been described for the bacterial enzymes CloQ and NphB, previously (Kuzuyama *et al.*, 2005; Metzger *et al.*, 2010). Consequently, a common origin has been suggested for the enzymes of the DMATS superfamily and of the CloQ/NphB group (Bonitz *et al.*, 2011).

1.5. Chemoenzymatic synthesis of prenylated aromatic compounds

As described previously, the biological and pharmacological activities of many secondary metabolites depend on their prenylation and their improved interaction with biomembranes and proteins (Botta *et al.*, 2005). This has also been discovered in cytotoxicity experiments with diverse stereoisomers of prenylated cyclic dipeptides (Wollinsky *et al.*, 2012a). Therefore, the regiospecific production of unnatural prenylated compounds is of interest for current research. Such compounds can be obtained by using aromatic prenyltransferases from fungi and bacteria with alternative substrates via chemoenzymatic synthesis.

The fungal L-tryptophan prenyltransferases FgaPT2, 5-DMATS and 7-DMATS catalyzed the regiospecific regular C4-, C5- and C7-prenylation of several simple indole derivatives in the presence of DMAPP, respectively (Kremer and Li, 2008; Steffan *et al.*, 2007; Yu *et al.*, 2012b). The bacterial prenyltransferases IptA and its homologues 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ catalyzed the formation of several C6-prenylated indole derivatives (Takahashi *et al.*, 2010; Winkelblech and Li, 2014). The latter two enzymes also utilized GPP as prenyl donor (Winkelblech and Li, 2014). Furthermore, the significantly modified indole derivatives DMA-indoleacetonitriles and DMA-indolocarbazoles, e.g. 3-DMA-7-hydroxy-K252c (**30**), were obtained in vitro using purified recombinant bacterial and fungal prenyltransferases, respectively (Ozaki *et al.*, 2013; Yu *et al.*, 2012a).



3-DMA-7-hydroxy-K252c (30) | **7-farnesyl-1,7-dihydroxy-6-methylxanthone (31)** | **6-DMA-2,3-dihydroxynaphthalene (32)** | **2'-DMA-hesperetin (33)**

Figure 1.10 Examples of different chemoenzymatically produced prenylated aromatic compounds.

Cyclic dipeptide prenyltransferases in turn can be used as biocatalyst for the formation of different prenylated dipeptides. For FtmPT1 not only C2-regular, but also C3-regular prenylated tryptophan-containing cyclic dipeptides have been described (Wollinsky *et al.*, 2012b). Recent investigations have also shown that FtmPT1 is able to prenylate a non-aromatic

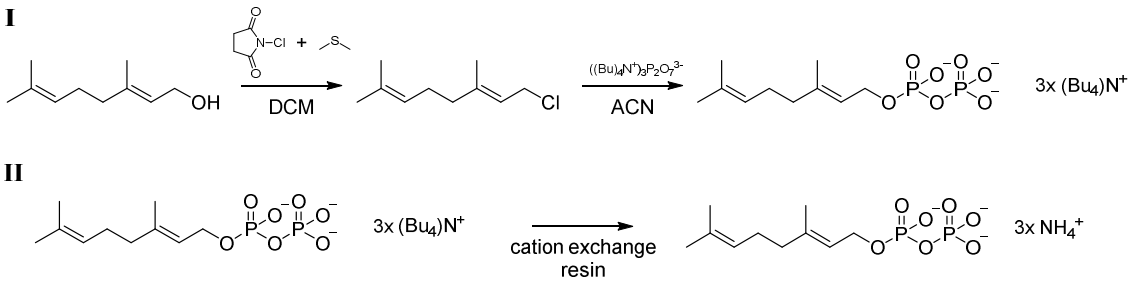
carbon atom using the indole derivative indolylbutenone (Chen *et al.*, 2012). Comparative investigation on AnaPT, CdpNPT and CdpC3PT revealed that these enzymes produced stereospecifically C3-prenylated cyclic dipeptides (Yin *et al.*, 2009a; Yu *et al.*, 2013).

Not only compounds containing an indolyl moiety, but also other aromatic compounds could be used as substrates. L-tyrosine and derivatives can be prenylated by the L-tyrosine prenyltransferases SirD (Rudolf and Poulter, 2013; Zou *et al.*, 2010) and TyrPT (Fan *et al.*, 2014). Recent studies on the xanthone prenyltransferase XptB and the benzoquinone prenyltransferase AstPT broadened the availability of O-prenylated, O-geranylated and O-farnesylated xanthones, e.g. 7-farnesyl-1,7-dihydroxy-6-methylxanthone (**31**) (Pockrandt *et al.*, 2012; Tarcz *et al.*, 2014b). C- as well as O-, mono- and di-prenylated hydroxynaphthalenes, e.g. 6-DMA-2,3-dihydroxynaphthalene (**32**), were also formed using the L-tryptophan prenyltransferases or cyclic dipeptide prenyltransferases (Winkelblech and Li, 2014; Yu *et al.*, 2011). Prenyl and geranyl moieties have been attached to different flavonoid substrates, e.g. 2'-DMA-hesperetin (**33**), in the presence of diverse prenyltransferases such as 7-DMATS, NphB and NovQ (Kumano *et al.*, 2008; Ozaki *et al.*, 2009; Yu and Li, 2011).

1.6. Chemical synthesis of prenyl diphosphates

For in vitro investigations of the prenylation reactions of prenyltransferases, several components including the prenyl diphosphates are needed. To obtain the desired diphosphate different chemical synthesis protocols can be applied. The prenyl analogues used in this thesis have been synthesized following a protocol by Woodside *et al.* (1988). The general procedure of this synthesis beginning with the corresponding alcohol and yielding a trisammonium geranyl diphosphate is described below (Scheme 1.3).

Scheme 1.3 Schematic presentation of the chemical synthesis of geranyl diphosphate from geraniol (modified from Woodside et al., 1988).



I: chemical reaction steps from alcohol to diphosphate; II: exchange step of the diphosphate counterion (DCM = dichloromethane, ACN = acetonitrile).

The first step in this diphosphate synthesis is the chlorination of geraniol using an *N*-chlorosuccinimide (NCS)-dimethyl sulfide (DMS)-complex. As described by Woodside *et*

al., the three reagents are added to dichloromethane (DCM) at -30 to -40 °C. Due to the thermolability of the NCS-DMS complex, the reaction is then kept at 0 °C. From the NCS-DMS complex and the allyl alcohol an allyl-sulfoxonium is formed, which is then subsequently chlorinated by the free chloride anion derived from NCS (Scheme 1.3; I) (Corey *et al.*, 1972).

After removal of NCS and DMS and related byproducts by extraction with water and pentane, the organic phase (DCM/pentane) is concentrated and the residue directly used for the next reaction step. In this step, geranyl chloride reacts with tris-tetrabutylammonium (TBA) hydrogen diphosphate (preparation see also Woodside *et al.*) in acetonitrile at room temperature to form the geranyl diphosphate-TBA salt in a direct displacement reaction (Scheme 1.3; I) (Dixit *et al.*, 1981). As the TBA could likely interfere in later enzymatic reactions, it has to be exchanged with a smaller cation. This is achieved by funneling the concentrated reaction product over an ammonium equilibrated cation exchange resin in an aqueous ammonium buffer. The end-product geranyl diphosphate ammonium salt is obtained after extraction with ACN/isopropanol and after solvent evaporation. For our subsequent experiments, further purification over cellulose column, as described by Woodside *et al.*, was not necessary.

1.7. Prenyl diphosphate analogues

Modification of the prenyl moiety itself has been of longstanding interest to understand and regulate the mechanisms of prenyltransferases. Over 40 years ago, experiments to determine the reactivity range of a DMAPP prenyltransferase and a FPP synthase towards simple branched or elongated isoprene analogues have already been conducted (Nishino *et al.*, 1972; Popjak *et al.*, 1969). Further studies on farnesyl synthases included the synthesis and investigation on the acceptance of cyclohexane containing DMAPP analogues (Nagaki *et al.*, 2009a), of ether derivatives (Nagaki *et al.*, 2009b) and of chloride substituted IPP and DMAPP analogues (Heaps and Poulter, 2011). Due to the involvement of prenylation for oncogenic Ras protein activity (Berndt *et al.*, 2011; Kato *et al.*, 1992), possible inhibitors in form of the farnesyl analogues have been of interest in diverse studies. Methylfluorinated FPP analogues (Dolence and Poulter, 1995; Dolence and Poulter, 1996), an unsaturated FPP analogue (Liu and Prestwich, 2004) and substituted aromatic anilinogeranyl diphosphates (AGPP) (Roberts *et al.*, 2006) have been synthesized and tested. Additional synthetic approaches yielded derivatives of the aforementioned AGPPs with the amino group exchanged with other heteroatoms such as thiol, oxygen, methoxy or methyl groups. (Subramanian *et al.*, 2008). Farnesyl analogues with aryl instead of DMA moieties have been synthesized and used as inhibitors or substrates for farnesyltransferases (Subramanian *et al.*, 2012).

The synthesis and usage of DMAPP analogues was also of interest in the functional elucidation of other transferases. To determine the function of isopentenyl diphosphate isomerases, analogues containing tertiary nitrogen, epoxid, cylcopropyl, allene or alkyne moieties have been synthesized and evaluated (Muehlbacher and Poulter, 1988; Sharma *et al.,* 2010; Walker *et al.,* 2008). Different DMAPP analogues have been applied to characterize the reactivity of an aspulvinone DMA transferase (Takahashi *et al.,* 1978). The catalytic mechanism of CymD has been further examined with assistance of fluorinated as well as isotopic labeled DMAPP (Qian *et al.,* 2012). Furthermore, an electrophilic aromatic substitution catalyzed by 4-DMATS was postulated using *E/Z* mono- and di-methylfluorinated DMAPP analogues in the prenyltransfer reaction (Gebler *et al.,* 1992).

The search for suitable alkyl donor analogues also led to the studies of DNA- and protein-methyltransferases (DNA-MTases, PMTases). The natural methyl donor of these enzymes is *S*-adenosyl-L-methionine (SAM) (Struck *et al.,* 2012). Investigations on iodoethyl substituted (mustard gas like) analogues have shown that these substances bind covalently to DNA and proteins (Osborne *et al.,* 2007; Weller and Rajski, 2006). DNA alkylation with alkyl, allyl and propargyl or amino-allyl analogues, for sequence specific DNA derivatization and labeling purposes has also been described (Dalhoff *et al.,* 2006; Lukinavicius *et al.,* 2007). Comparable results have been obtained with allyl and propargyl analogues for diverse PMTases, leading to alkylated protein derivatives accessible to "clicklabeling" (Islam *et al.,* 2011; Wang *et al.,* 2011). More recently, the broader acceptance of allyl *Se*-adenosyl-L-methionine (SeAM) by several PMTases has been observed and these analogues could be produced in multi gram scale (Bothwell *et al.,* 2012; Bothwell and Luo, 2014). The successful biochemical production of SAM and SeAM analogues and subsequent utilization by methyltransferases was proven as a suitable alternative to synthetic approaches (Singh *et al.,* 2014; Thomsen *et al.,* 2013). Finally, SAM analogues have been used in the formation of alternate coumarin derivatives as precursors of aminocoumarin antibiotics (Stecher *et al.,* 2009).

In this thesis, alkyl, allyl and benzyl analogues of DMAPP were synthesized and tested towards a series of prenyltransferases of the DMATS superfamily. The corresponding products have been isolated and the structures were elucidated.

2. Aims of this thesis

The following issues have been addressed in this thesis:

Biochemical investigations on the acceptance of alkyl and allyl DMAPP analogues by L-tryptophan prenyltransferases and characterization of enzymatic products.

Prenyltransferases of the DMATS superfamily show broad substrate promiscuity, but high prenyl donor specificity. The aim of this thesis was to elucidate the behavior of L-tryptophan prenyltransferases, with distinct regiospecificity, towards slightly modified DMAPP analogues and to determine the importance of the double bond and its position for an acceptance by these enzymes. The following experiments were carried out:

- Synthesis of alkyl and allyl DMAPP analogues.
- Overproduction of FgaPT2, 5-DMATS and 7-DMATS in *E. coli* and subsequent purification.
- Incubation of assays containing L-tryptophan and DMAPP analogues in the presence of FgaPT2, 5-DMATS and 7-DMATS.
- Analysis of the enzyme activities and isolation of the enzyme products.
- Structure elucidation of the products by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) analysis.
- Determination of the kinetic parameters of the corresponding reactions.

Biochemical investigations on the acceptance of alkyl and allyl DMAPP analogues by cyclic dipeptide prenyltransferases and characterization of enzymatic products.

It has been shown that L-tryptophan prenyltransferases catalyze the transfer reaction of a monomethylallyl as well as a 2-pentenyl moiety onto L-tryptophan. Depending on the allyl donor, the position of the allyl residue is shifted partially or completely, compared to the reaction with DMAPP. Consequently, we were intrigued to examine the reaction of tryptophan-containing cyclic dipeptide prenyltransferases towards both allyl diphosphates. Emphasis was put on the comparison of the natural reactions of the used prenyltransferases, i.e. C3- and C2-prenylation as well as C2-reverse and regular prenylation. The following experiments were carried out:

- Overproduction of AnaPT, CdpNPT, CdpC3PT, FtmPT1 and BrePT in *E. coli* and subsequent purification

- Incubation of assays containing respective natural or best-accepted substrates and DMAPP analogues in the presence of AnaPT, CdpNPT, CdpC3PT, FtmPT1 and BrePT.
- Analysis of the enzyme activities and isolation of enzyme products.
- Structure elucidation of the products by NMR and MS analyses.
- Determination of the kinetic parameters of the corresponding reactions.

Synthesis of a benzyl diphosphate and proof of the biochemical transfer onto L-tryptophan and derivatives thereof

L-tryptophan and tryptophan-containing cyclic dipeptides were successfully alkylated with two simple allyl DMAPP analogues in the presence of several enzymes of the DMATS superfamily. Further investigation focused on a more bulky analogue of DMAPP. Benzyl diphosphate represented a suitable donor for the previously used prenyltransferase FgaPT2. The following experiments were carried out:

- Synthesis of benzyl-PP.
- Overproduction of several indole prenyltransferases in *E. coli* and subsequent purification.
- Incubation of assays containing respective natural or best-accepted substrates and DMAPP analogues in the presence of several indole prenyltransferases.
- Analysis of the enzyme activity and isolation of enzyme products of FgaPT2.
- Structure elucidation of the products by NMR and MS analyses.
- Determination of the kinetic parameters of the corresponding reactions.

Biochemical investigations on prenyltransferases catalyzing C6- and C7-prenylations towards unnatural allyl and benzyl diphosphates.

Successful transfer of unnatural allyl diphosphates has been described using prenyltransferases for various positions of the indole ring, with the exception of C6- and C7-prenyltransferases. Recently, two *Streptomyces* 6-DMAT synthases and a L-tyrosine O-prenyltransferase (TyrPT), with L-tryptophan C7-prenyltransferase activity were elucidated biochemically. These additional enzymes allowed us to further investigate the unnatural alkylation of L-tryptophan. To compare the results of the bacterial 6-DMATS with the fungal 5-DMATS, a bacterial 5-DMATS was elucidated additionally. The following experiments were carried out:

- Cloning and overproduction of the recombinant 5-DMATS SCO74567 from *Streptomyces* in *E. coli* and subsequent purification.

- Overproduction of the recombinant proteins of the two C-6 prenyltransferases 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ as well as the L-tryptophan C7-prenylating TyrPT in *E. coli* and subsequent purification.
- Incubation of assays containing L-tryptophan and DMAPP analogues in the presence of 5-DMATS, 6-DMATS$_{Sa}$, 6-DMATS$_{Sv}$ and TyrPT.
- Analysis of the enzyme activity and isolation of enzyme products.
- Structure elucidation of the products by NMR and MS analyses.
- Determination of the kinetic parameters of the corresponding reactions.

3. Results and discussion

3.1. Biochemical investigations on alkyl and allyl DMAPP analogues in the presence of fungal L-tryptophan prenyltransferases

Indole prenyltransferases catalyzed regiospecific prenylations on simple indole derivatives and tryptophan-containing cyclic dipeptides (Kremer and Li, 2008; Steffan *et al.*, 2007; Yu *et al.*, 2012b). Likewise, structures of higher complexity such as flavonoids, hydroxynaphthalenes, indolocarbazoles, xanthones and tetracyclic polyketides were also converted by these enzymes to prenylated derivatives (Chooi *et al.*, 2012; Sanchez *et al.*, 2011; Yu *et al.*, 2012a; Yu *et al.*, 2011; Yu and Li, 2011). These findings revealed a low specificity towards substrates of different substance classes for several prenyltransferases of the DMATS superfamily.

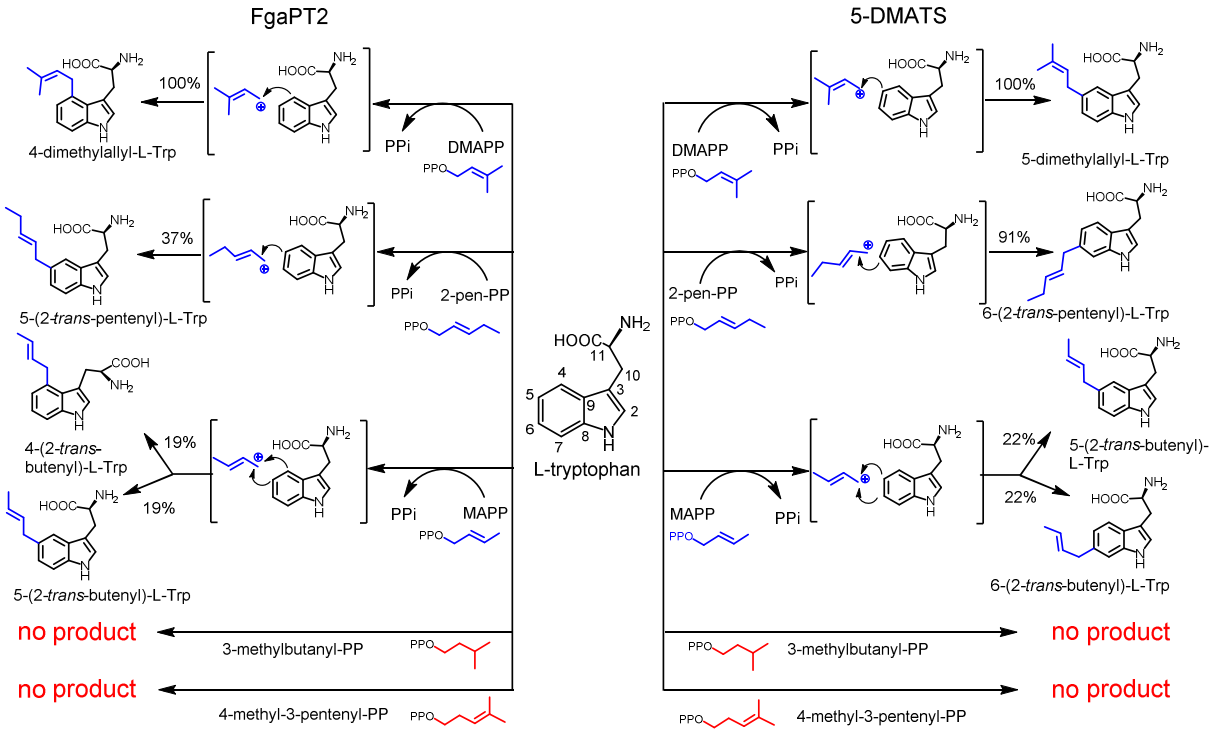
The prenylation reactions catalyzed by these enzymes are Friedel–Crafts alkylations (Gebler *et al.*, 1992; Heide, 2009a) and represent a useful biochemical tool for the creation of a multitude of diversely alkylated aromatic compounds. However, a broad usage of these enzymes is limited by their high prenyl donor specificity. The enzymes of the DMATS superfamily usually accepted solely DMAPP as prenyl donor (Yu and Li, 2012). Only recently, the acceptance of GPP and FPP by DMAPP utilizing indole prenyltransferases has been demonstrated (Pockrandt *et al.*, 2014; Pockrandt and Li, 2013; Tarcz *et al.*, 2014b; Winkelblech and Li, 2014). Another exception is VrtC, which uses GPP as natural isoprene substrate (Chooi *et al.*, 2012). This donor specificity motivated us to synthesize and test altered DMAPP structures in the presence of L-tryptophan prenyltransferases.

Following the synthesis of GPP described by Woodside *et al.* (1988), four DMAPP analogues were synthesized. Two carried modifications influencing their allylic nature, i.e. either by deleting the double bond or by shifting it to the γ-position via introduction of a methylene group between diphosphate and the double bond. Other modifications targeted the methyl groups of the structure, either by deleting or by shifting of one methyl group to the end of the allyl chain (Scheme 3.1). The analogues were tested with L-tryptophan as aromatic substrate in the presence of the previously purified octahistidine (His_8)-tagged protein FgaPT2 as well as the hexahistidine (His_6)-tagged proteins 5-DMATS and 7-DMATS. Interestingly, none of the used enzymes displayed conversion with the double bond modified analogues. Based on these results, it is proposed that the existence of the double bond at β-position is essential for the enzymatic catalysis. Furthermore, product formation was only observed in the presence of FgaPT2 and 5-DMATS, but not in that of 7-DMATS. A strong influence of the regiospecificity

by the alkylation agents was also detected. Monomethylallyl diphosphate (MAPP) was well accepted by FgaPT2 and 5-DMATS. Both enzymes showed even higher activities with 2-pentenyl diphosphate (2-pentenyl-PP) than with MAPP. To elucidate their structures, the enzyme products were isolated on HPLC and analyzed by NMR and MS analyses. MS data proved the monoalkylation of all of the isolated compounds. The NMR spectra revealed the products that the products were regular C5-alkylated and C6-alkylated L-tryptophan with 2-pentenyl-PP as allyl donor in the presence of FgaPT2 and 5-DMATS, respectively. Using MAPP as allyl donor, FgaPT2 produced a mixture of regular C4- and C5-alkylated and 5-DMATS a mixture of regular C5- and C6-alkylated L-tryptophan, respectively (Scheme 3.1).

These results demonstrated clearly the shifts of the regioselectivity in the presence of unnatural allyl donors. The kinetic parameters of both analogues, i.e. K_M and k_{cat} values, were determined and compared to the respective values for DMAPP to classify their biochemical properties. 2-pentenyl-PP and MAPP showed weaker affinity towards FgaPT2 with K_M values around ten to twelve times higher, compared to DMAPP. The K_M values of 2-pentenyl-PP were two times higher while the value of MAPP was around half that of DMAPP in the case of 5-DMATS and therefore showed an insignificant preference towards DMAPP and its analogues. Turnover numbers, on the other hand, were relatively low for both analogues. Nonetheless, these DMAPP analogues could be useful allyl donors for chemoenzymatic synthesis catalyzed by enzymes of the DMATS superfamily.

Scheme 3.1 Unnaturally alkylated products obtained in the presence of diverse L-tryptophan prenyltransferases (modified from Liebhold et al., 2012).



For details on this work, please see the publication (section 4.1)

Mike Liebhold, Xiulan Xie and Shu-Ming Li (2012) Expansion of enzymatic Friedel–Crafts Alkylation on Indoles: Acceptance of unnatural β-unsaturated Allyl Diphosphates by DMAT Synthases. *Organic Letters* 14: 4882–4885.

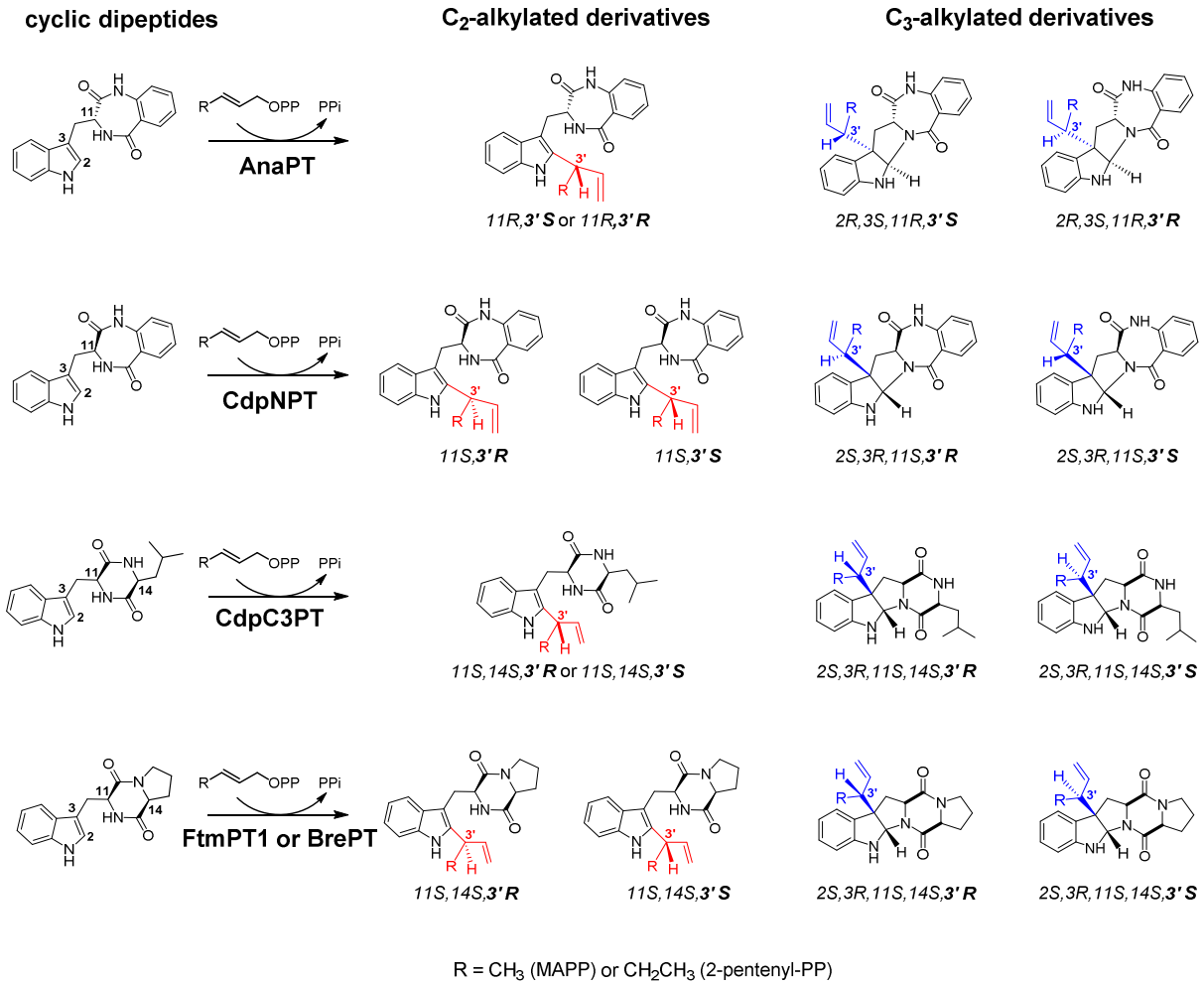
3.2. Biochemical investigations on alkyl and allyl DMAPP analogues in the presence of fungal tryptophan-containing cyclic dipeptide prenyltransferases

As described previously, prenyltransferases of the DMATS superfamily are versatile enzymes accepting a large number of aromatic substrates with high specificity towards prenyl donors (Yu and Li, 2012). The chemoenzymatic attachment of the prenyl moiety onto their natural substrates, such as L-tryptophan and tryptophan-containing cyclic dipeptides, is highly regiospecific and can occur on seven positions of the indole ring system (Li, 2010; Yu and Li, 2012). Cyclic dipeptide prenyltransferases, e.g. His_6-tagged AnaPT, CdpNPT, CdpC3PT usually catalyze reverse C3-prenylation of the indole moiety of the cyclic dipeptide, while His_6-tagged FtmPT1 and pentahistidine (His_5)-tagged BrePT catalyze regular and reverse C2-prenylation, respectively (Grundmann and Li, 2005; Schuller *et al.*, 2012; Yin *et al.*, 2013a; Yin *et al.*, 2009b; Yin *et al.*, 2010). As demonstrated above, two allyl diphosphate analogues of DMAPP were successfully used by the L-tryptophan prenyltransferases FgaPT2 and 5-DMATS (Liebhold *et al.*, 2012). This inspired us to test these DMAPP analogues towards cyclic dipeptide prenyltransferases.

At first, the DMAPP analogues 2-pentenyl-PP and MAPP were incubated in the presence of the C3-prenyltransferases AnaPT, CdpNPT and CdpC3PT together with their natural or best accepted substrates. All three enzymes produced multiple compounds in moderate to high yields. The products were isolated on HPLC using reverse phase 18 or silica gel columns and subjected to NMR and MS analyses. It was shown that both, reverse C2- and C3-alkylated derivatives were formed in different ratios. The products containing the 2-pentenyl and monomethylallyl moiety retained their overall stereochemistry, i.e. α-orientation for AnaPT and β-orientation for CdpC3PT and CdpNPT, as observed for DMAPP. With two exceptions, the introduction of a new stereo center at C-3', led to the formation of all possible C-3'-stereoisomers (Scheme 3.2). To further investigate the behavior of cyclic dipeptide prenyltransferases towards DMAPP analogues, the two regular and reverse C2-prenyltranferases, i.e. FtmPT1 and BrePT, were tested, respectively. Structure elucidation of the multiple products yielded comparable results, as acquired for the C3-prenyltransferases.

Both enzymes catalyzed the formation of C2- and C3-alkylated diastereomers (Scheme 3.2). Thus, allyl DMAPP analogues completely break the regiospecificity of these enzymes. Furthermore, the ratios of the detected products strongly depend on the combination of enzyme, substrate and used alkyl donor. To study the biochemical behavior, K_M and k_{cat} values were determined for each enzyme-donor combination. While these enzymes showed similar or even higher affinity in comparison to DMAPP, the k_{cat} values were much lower than that of DMAPP. These results provide evidence for a common catalytic ability of C-2 and C-3 cyclic dipeptide prenyltransferases in the presence of unnatural alkyl donors.

Scheme 3.2 C2- and C3-reverse alkylated products obtained in the presence of diverse cyclic dipeptide prenyltransferases (modified from Liebhold et al., 2013).



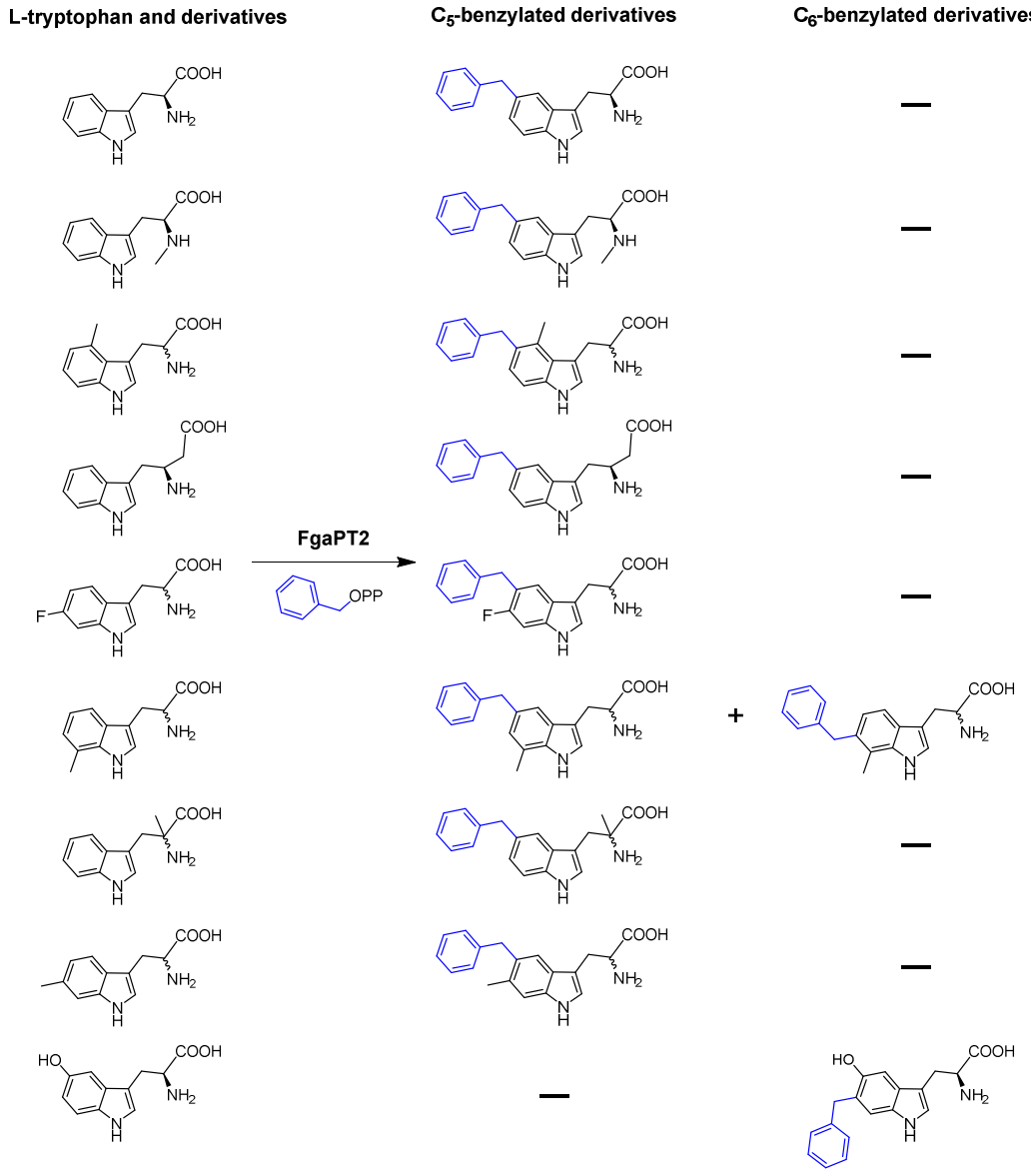
For details on this work, please see the publication (section 4.2)

Mike Liebhold, Xiulan Xie and Shu-Ming Li (2013). Breaking cyclic dipeptide prenyltransferase regioselectivity by unnatural alkyl donors. *Organic Letters* 15: 3062–3065.

3.3. Biochemical investigations on a benzyl DMAPP analogue in the presence of a fungal L-tryptophan prenyltransferase

As demonstrated above, investigations on L-tryptophan and cyclic dipeptide prenyltransferases showed the successful alkylation of their respective substrates with unnatural DMAPP analogues (Liebhold *et al.*, 2012; Liebhold *et al.*, 2013). Similar alkenyl and also alkinyl as well as aryl moieties were transferred onto proteins or aromatic compounds via SAM depended methyltransferases (Islam *et al.*, 2011; Stecher *et al.*, 2009; Wang *et al.*, 2011). Our preliminary work encouraged us to examine several indole prenyltransferases of the DMATS superfamily towards benzyl diphosphate (benzyl-PP).

Scheme 3.3 Benzylated products obtained from L-tryptophan derivatives in the presence of FgaPT2 (modified from Liebhold and Li, 2013).



After successful synthesis of benzyl-PP, we initially tested its acceptance by three L-tryptophan prenyltransferases (FgaPT2, 5-DMATS, 7-DMATS) as well as six cyclic dipeptide prenyltransferases (AnaPT, CdpNPT, CdpC3PT, CTrpPT, FtmPT1, BrePT) in the presence of their respective natural or best accepted substrates. Five enzymes accepted benzyl diphosphate as unnatural donor. FgaPT2 showed the highest activity with a yield of about 60%, whereas the yield for the other enzymes was very low. Therefore, further experiments were carried out with FgaPT2. In addition to L-tryptophan, 25 other simple indole derivatives were assayed with benzyl-PP. For 16 L-tryptophan derivatives including several substrates with a substitution on the indole ring, product formation was clearly observed. The products of L-tryptophan and eight derivatives thereof showed yields of more than 10% and were isolated on HPLC. Their structures were elucidated by NMR and MS analyses. FgaPT2 showed activity with 5-hydroxy-L-tryptophan, but not other C5-substituted derivatives. The NMR spectra unequivocally revealed, with two exceptions, the C5-benzylation of the accepted substrates (Scheme 3.3). In the case of 5-hydroxy-L-tryptophan and 7-methyl-L-tryptophan, a C6-benzylated compound was observed as sole and as additional product, respectively (Scheme 3.3). For biochemical characterization, the K_M and k_{cat} values for L-tryptophan and benzyl-PP were determined. Although FgaPT2 showed a much lower affinity to benzyl-PP than to DMAPP (Unsöld and Li, 2005), the acceptance of this structurally more complex DMAPP analogue makes FgaPT2 into an interesting biocatalyst for chemoenzymatic synthesis.

For details on this work, please see the publication (section 4.3)

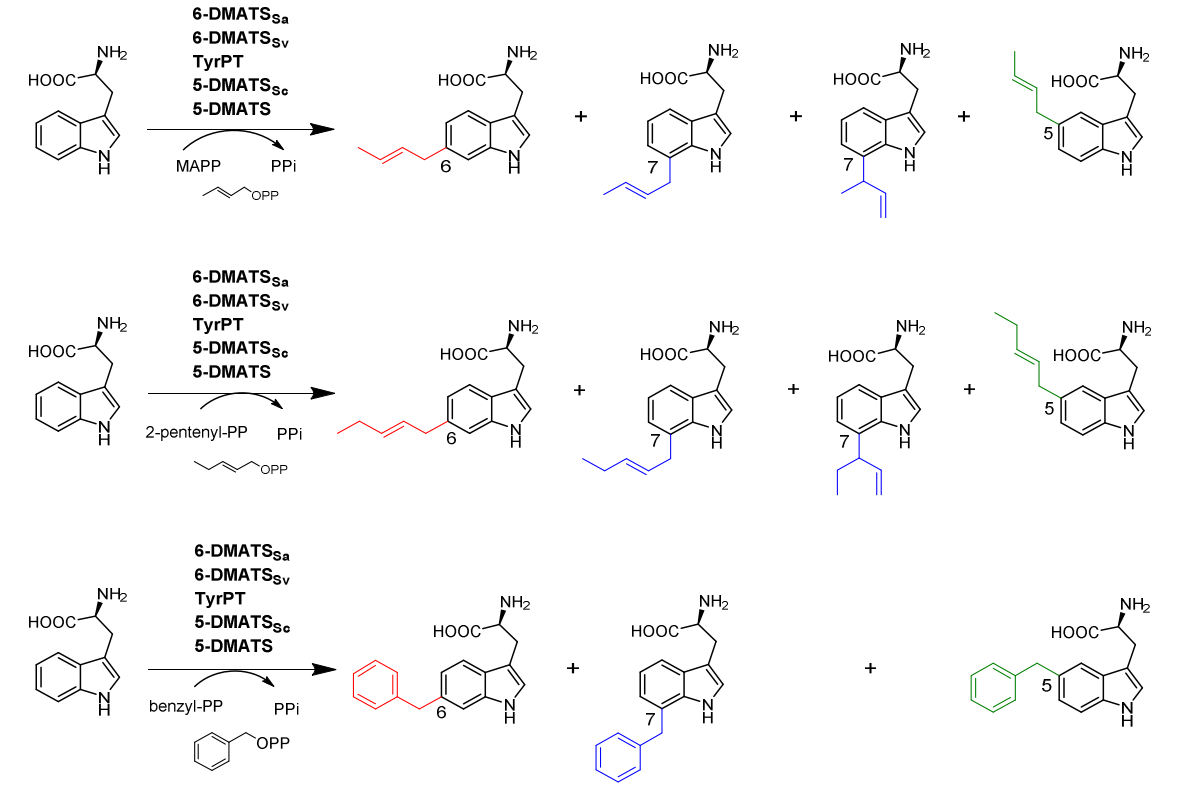
Mike Liebhold and Shu-Ming Li (2013) Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase. *Organic Letters* 15: 5834–5837.

3.4. Biochemical investigations on microbial enzymes catalyzing C5-, C6- and C7-prenylations of L-tryptophan towards unnatural allyl and benzyl diphosphates

At the beginning of this thesis, we investigated the acceptance of a 4-DMATS (FgaPT2) and a 5-DMATS from fungi towards unnatural alkyl donors (Liebhold *et al.*, 2012). In a recent study, the biochemical properties of two bacterial 6-DMAT synthases were characterized (Winkelblech and Li, 2014) and with TyrPT a new fungal tryptophan C7-prenylating enzyme has also been investigated (Fan *et al.*, 2014). These enzymes enabled us to complete our studies on DMAPP analogues in the presence of C-6 and C-7 prenylating enzymes.

Firstly, we examined the behavior of the two bacterial C6-prenyltransferases 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ with MAPP, 2-pentenyl-PP and benzyl-PP as allyl or benzyl donors and L-tryptophan as acceptor. Both enzymes accepted all three DMAPP analogues. After isolation on HPLC, subsequent analyses by MS and ^{1}H-NMR revealed regular C6-monoalkylated/benzylated compounds as sole product for all enzyme assays. In following experiments, the fungal tryptophan C7-prenylating enzyme TyrPT was incubated with the unnatural DMAPP analogues. MS and ^{1}H-NMR analyses showed that three to four monoalkylated/benzylated compounds were obtained. In addition to the predominant regular C6-alkylated/benzylated compounds, regular and reverse C7- as well as regular C5-alkylated L-tryptophan was formed. To further elucidate this divergent behavior the bacterial C5-prenyltransferase 5-DMATS$_{Sc}$ (SCO7467) was overexpressed and purified as described by Osaki *et al.* in 2013, and then tested towards the DMAPP analogues in the presence of L-tryptophan. Structure elucidation again revealed C6-alkyl/benzyl-L-tryptophan as one of the predominant products and C7- as well as C5-alkyl/benzyl-L-tryptophan as side products.

Scheme 3.4 Alkylated and benzylated products obtained in the presence of microbial tryptophan C5, C6 and C7-prenylating enzymes (modified from Winkelblech *et al.*, 2014 (submitted manuscript)).



6-DMATS$_{Sa}$: 6-DMATS from *S. ambofaciens* 5-DMATS: 5-DMATS from *A. clavatus* TyrPT: from *A. niger* with a C7-prenyltransferase activity
6-DMATS$_{Sv}$: 6-DMATS from *S. violaceusniger* 5-DMATS$_{Sc}$: 5-DMATS from *S. coelicolor*

For better comparison, the results for 5-DMATS (Liebhold and Li, 2013) were reproduced and supplemented with the data obtained from incubation mixtures of 5-DMATS with benzyl-PP,

which yielded C6- and C5-benzyl-L-tryptophan as main and side product, respectively. Taking all data together, 11 alkylated/benzylated L-tryptophan derivates were isolated from the incubation mixtures of the DMAPP analogues and L-tryptophan in the presence of the five tryptophan prenylating enzymes (Scheme 3.4). The K_M and k_{cat} values for all DMAPP analogues in the presence of the different enzymes were determined. The K_M values showed a relatively high affinity of the tested enzymes towards MAPP, 2-pentenyl-PP and benzyl-PP. The k_{cat} values for all enzyme-donor combinations on the other hand were much lower than those of DMAPP.

The aforementioned data show, that C-6 of the indole ring is the favored position for the biocatalytic attachment of the unnatural alkyl/benzyl moieties in the presence of the tested tryptophan C5-, C6- and C7-prenylating enzymes.

For details on this work, please see the manuscript (section 4.4)

Julia Winkelblech,*** Mike Liebhold,*** Xiulan Xie and Shu-Ming Li (2013) Unnatural DMAPP Analogues turn C-6 into the Preferable Alkylation/Benzylation Position for Tryptophan C5-, C6- and C7-Prenylating Enzymes. *Organic Letters* (submitted) [*equal contribution]

4. Publications

4.1. Expansion of Enzymatic Friedel–Crafts Alkylation on Indoles: Acceptance of Unnatural β-unsaturated Allyl Diphospates by Dimethylallyl-tryptophan Synthases

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Expansion of Enzymatic Friedel–Crafts Alkylation on Indoles: Acceptance of Unnatural β-Unsaturated Allyl Diphospates by Dimethylallyl-tryptophan Synthases

Mike Liebhold,[†] Xiulan Xie,[§] and Shu-Ming Li*[,†]

Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17a, 35037 Marburg, Germany, and Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany

shuming.li@staff.uni-marburg.de

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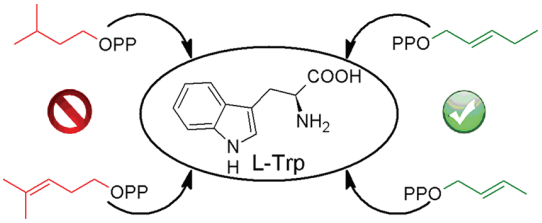
ABSTRACT



Prenyltransferases of the dimethylallyl-tryptophan synthase (DMATS) superfamily catalyze Friedel–Crafts alkylation with high flexibility for aromatic substrates, but the high specificity for dimethylallyl diphosphate (DMAPP) prohibits their application as biocatalysts. We demonstrate here that at least one methyl group in DMAPP can be deleted or shifted to the δ-position. For acceptance by some DMATS enzymes, however, a double bond must be situated at the β-position. Furthermore, the alkylation position of an analogue can differ from that of DMAPP.

Prenyltransferases are a large family of enzymes found in many living organisms such as bacteria, fungi, and plants, which are responsible for transfer of a prenyl moiety (nxC5) to another prenyl residue, a protein, or an aromatic substrate.[1] The reactions catalyzed by the last enzyme group, known as "aromatic prenyltransferases", are often Friedel–Crafts alkylations on different aromatic systems such as indoles, naphthalenes, flavonoids, or phenylpropanoids, resulting in the formation of biologically active metabolites.[1,2] One special group of the aromatic prenyltransferases shares a significant sequence similarity with dimethylallyl-tryptophan synthase (DMATS) from *Claviceps*,[3] and they are therefore classified as enzymes of the DMATS superfamily.[2a] The members of this family are involved in the biosynthesis of diverse important natural products including ergot alkaloids and usually catalyze the transfer of a dimethylallyl moiety from dimethylallyl diphosphate (DMAPP) to different positions of the indole ring of L-tryptophan and derivatives thereof.[1c] They showed high flexibility toward their aromatic substrates and accepted even hydroxynaphthalenes, flavonoids, xanthones, and tetracyclic napthacenediones as prenylation substrates.[2b,c,4] In contrast, these enzymes are usually highly specific toward DMAPP and did not accept other prenyl diphosphates as prenyl donors.[2a] One exception was found

[†] Institut für Pharmazeutische Biologie und Biotechnologie.
[§] Fachbereich Chemie.

(1) (a) Heide, L. *Curr. Opin. Chem. Biol.* **2009**, *13*, 171–179. (b) Yazaki, K.; Tsurumaru, Y. *Phytochemistry* **2009**, *70*, 1739–1745. (c) Li, S.-M. *Nat. Prod. Rep.* **2010**, *27*, 57–78.

(2) (a) Yu, X.; Liu, Y.; Xie, X.; Zheng, X.-D.; Li, S.-M. *J. Biol. Chem.* **2012**, *287*, 1371–1380. (b) Yu, X.; Xie, X.; Li, S.-M. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 737–748. (c) Yu, X.; Li, S.-M. *ChemBioChem* **2011**, *12*, 2280–2283. (d) Ozaki, T.; Mishima, S.; Nishiyama, M.; Kuzuyama, T. *J. Antibiot.* **2009**, *62*, 385–92.

(3) Tsai, H. F.; Wang, H.; Gebler, J. C.; Poulter, C. D.; Schardl, C. L. *Biochem. Biophys. Res. Commun.* **1995**, *216*, 119–125.

(4) (a) Sanchez, J. F.; Entwistle, R.; Hung, J. H.; Yaegashi, J.; Jain, S.; Chiang, Y. M.; Wang, C. C.; Oakley, B. R. *J. Am. Chem. Soc.* **2011**, *133*, 4010–7. (b) Chooi, Y. H.; Wang, P.; Fang, J.; Li, Y.; Wu, K.; Wang, P.; Tang, Y. *J. Am. Chem. Soc.* **2012**, *134*, 9428–37.

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for the newly characterized DMATS-type prenyltransferase VrtC, which only utilizes GPP.[4b] This feature prohibits their usage as catalysts for Friedel–Crafts alkylation. We expand in this study their potential application for alkylation with unnatural alkyl diphosphates.

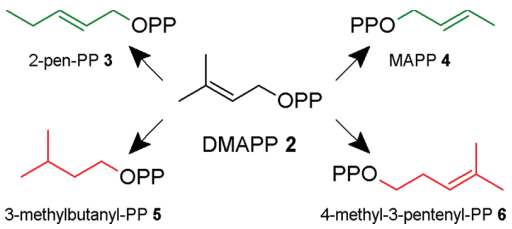


Figure 1. DMAPP and its analogues.

For this purpose, four DMAPP analogues (Figure 1) were successfully synthesized according to the method described for GPP by Woodside et al.,[5] which were confirmed by ^{1}H and ^{31}P NMR analyses. The four DMAPP analogues were used as alkyl donors for enzyme assays with FgaPT2 and 7-DMATS from *Aspergillus fumigatus* as well as with 5-DMATS from *Aspergillus clavatus*, which catalyzed prenylation of L-tryptophan at C-4, C-7, and C-5, respectively.[2a,6] Enzyme assays with DMAPP were used as positive controls (Figure 2). HPLC analysis of the incubation mixtures revealed an almost complete conversion of L-tryptophan to its prenylated products in the three enzyme assays with DMAPP after incubation with 5 μg of protein at 37 °C for 16 h (Figure 2). Shifting one methyl group of DMAPP from C-3 to C-4, as in the case of 2-pentenyl diphosphate (2-pen-PP), strongly reduced the activity of 7-DMATS. No product formation was detected in the incubation mixture under assay conditions (Figure 2C). In contrast, FgaPT2 and 5-DMATS still accepted 2-pen-PP well as a substrate (Figure 2A and 2B) and conversion yields of 37% and 91% from the respective assays with DMAPP were achieved under these conditions.

For structure elucidation, the enzyme products **3a** and **3b** were isolated from the incubation mixtures of FgaPT2 and 5-DMATS, respectively, and subjected to NMR and MS analyses. HR-MS data clearly showed the alkylation of both compounds by detection of $[M]^{+}$ ions at m/z 272.1518 (**3a**) and 272.1509 (**3b**), which are 68 Da larger than that of tryptophan.

In the ^{1}H NMR spectrum of **3a**, signals of a 2-pentenyl moiety proved the alkylation at a C-atom in a regular pattern.[2a]

The four aromatic protons of the indole moiety appeared, according to their chemical shifts and coupling pattern, in the same order as those of C5-prenylated indole derivatives obtained from enzyme assays of 5-DMATS

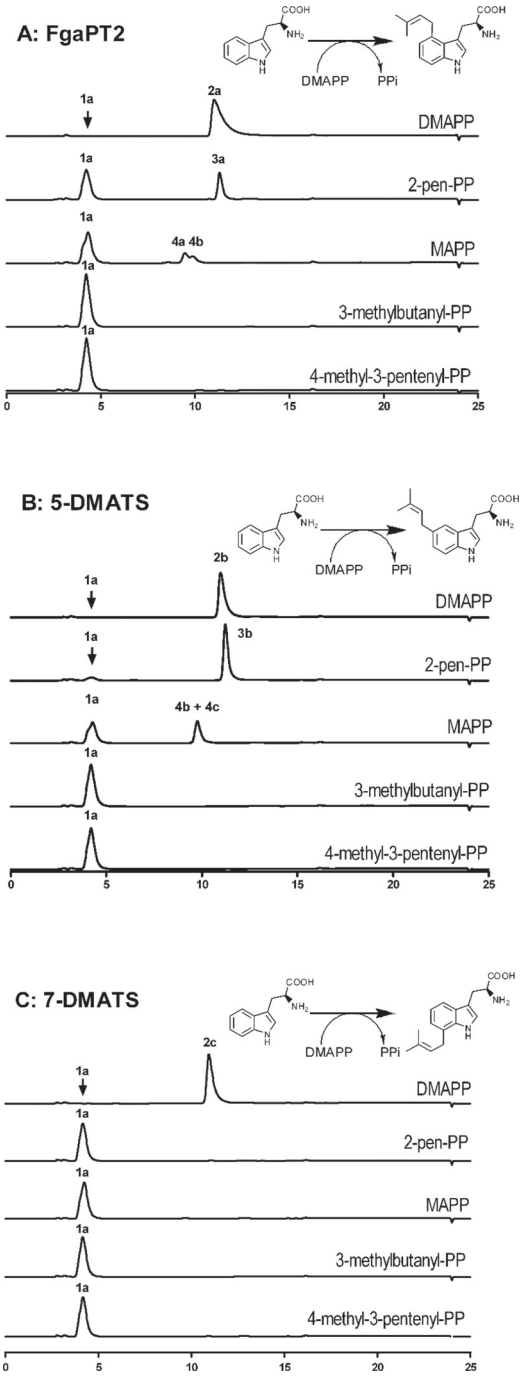


Figure 2. HPLC analysis of L-tryptophan after incubation with DMAPP or its analogues at 37 °C and 16 h.

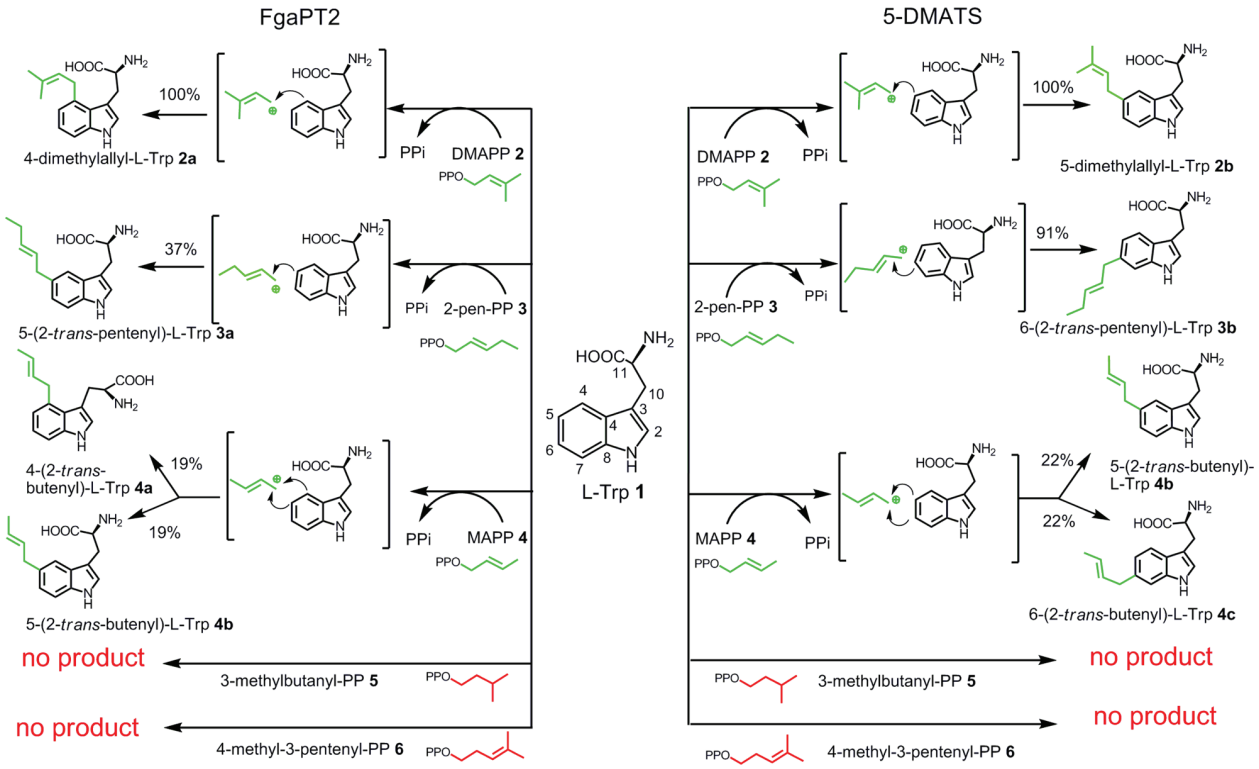
with DMAPP[2a] and therefore can be assigned to H-4, H-7, H-2, and H-6.

This indicated a C5-alkylation of L-tryptophan by FgaPT2 in the presence of 2-pen-PP and is somewhat unexpected for a C4-prenyltransferase with its natural substrate DMAPP (Scheme 1).[6b] Therefore, we obtained various 2D NMR spectra for **3a** to confirm the structure. In the HMBC spectrum, connectivities were clearly observed for H-4 of

(5) Woodside, A. B.; Huang, Z.; Poulter, C. D. *Org. Synth.* **1988**, *66*, 211–215.

(6) (a) Kremer, A.; Westrich, L.; Li, S.-M. *Microbiology* **2007**, *153*, 3409–3416. (b) Unsöld, I. A.; Li, S.-M. *Microbiology* **2005**, *151*, 1499–1505.

Scheme 1. Proposed Reaction Mechanisms and Enzyme Products of FgaPT2 and 5-DMATS with DMAPP and Analogues



the indole ring with C-3 and C-8 as well as with C-1′ of the alkyl moiety, proving unequivocally the C5-akylation of L-tryptophan in **3a**.

In the ^{1}H NMR spectrum of **3b**, similar signals to those of **3a** were also observed for a 2-pentenyl moiety. However, the ^{1}H NMR spectrum of **3b** differed clearly from that of **3a**, especially in the aromatic region, although the same number and similar coupling patterns were observed in both spectra. The signals of the four aromatic protons in **3b** corresponded very well to those of C6-prenylated indole derivatives obtained from enzyme assays of IptA with DMAPP.[7] This means that the alkylation of L-tryptophan by 5-DMATS was shifted from C-5 in the presence of DMAPP to C-6 in the presence of 2-pen-PP (Scheme 1).

The alkylation shifts from C-4 to C-5 for FgaPT2 and from C-5 to C-6 for 5-DMATS could be explained by the longer chain length and thus deeper extension of 2-pen-PP into the reaction chamber in comparison to that of DMAPP, so that C-1′ of the alkyl residue was closer to C-5 than C-4 in FgaPT2 and to C-6 than C-5 in 5-DMATS. To prove the influence of chain length of the alkyl diphosphate on the enzyme activity and alkylation position, the *cis*-methyl group of DMAPP was deleted to obtain methylallyl diphosphate (MAPP) (Figure 1), which was then used as an alkyl donor for enzyme assays of L-tryptophan with FgaPT2, 5-DMATS, and 7-DMATS. Again, 7-DMATS showed no detectable conversion (Figure 2C).

The enzyme activities of FgaPT2 and 5-DMATS were reduced, in comparison to those of DMAPP and 2-pen-PP, but still clearly detectable. Two and one product peaks were detected in the HPLC chromatograms of the incubation mixtures of L-tryptophan and MAPP with FgaPT2 and 5-DMATS, respectively. A conversion yield of 29% was calculated for FgaPT2, and 44%, for 5-DMATS, respectively. For structure elucidation, all three peaks were isolated and subjected to NMR and MS analyses. HR-MS data confirmed the presence of a butenyl moiety in their structures by detection of $[M+H]^+$ at m/z 259.1471 (**4a**) and 259.1434 (**4b**) or $[M]^+$ ion at 258.1388 (**4b**+**4c**), which are 54 Da larger than that of tryptophan.

The ^{1}H NMR spectra of **4a** and **4b** are similar to each other in the region of olefinic and aliphatic protons. Signals for a 2-*trans*-butenyl moiety were detected for both **4a** and **4b**. The spectra however differed clearly from each other by the chemical shifts and coupling pattern of the four aromatic protons in the spectrum of **4a** and **4b.**

The features of the aromatic protons of **4a** indicated an alkylation at C-4 or C-7. The HMBC spectrum clearly showed connectivities of H-1′ of the butenyl residue[8] to C-2′ (δ_C 131.2) and C-3′ (δ_C 125.0) as well as to C4 (δ_C 132.7) and C5 (δ_C 119.2) of the indole moiety. The alkylation position at C-4 of L-tryptophan was confirmed

(7) Takahashi, S.; Takagi, H.; Toyoda, A.; Uramoto, M.; Nogawa, T.; Ueki, M.; Sakaki, Y.; Osada, H. *J. Bacteriol.* **2010**, *192*, 2839–2851.

(8) The two protons at H-1′ of the butenyl residue differ from each other, probably due to the steric interaction with the side chain of tryptophan. δ_H 3.75 (1H, dd, 15.4, 6.3, H-1′) and δ_H 3.63 (1H, dd, 15.4, 6.3, H-1′).

by detection of additional connectivities in the indole moiety.[9]

The coupling pattern of the four aromatic protons in **4b** indicated a substitution at C-5 or C-6. The order of these coupling protons was found to be identical to those in **3a** and other C5-prenylated derivatives.[2a] The alkylation at C-5 of L-tryptophan in **4b** (Scheme 1) was also confirmed by the observation of connectivities in its HMBC spectrum.

Inspection of the ^{1}H NMR spectrum of the product peak isolated from the incubation mixture of L-tryptophan and MAPP with 5-DMATS revealed the presence of two compounds with a ratio of 1:1. Unfortunately, it was difficult to separate them from each other by HPLC under different conditions. Fortunately, it was yet possible to elucidate their structures based on the obtained spectra, because one of them can be easily identified as **4b** by comparison of their ^{1}H NMR spectra. The second one, **4c**, showed similar signals and identical order in the aromatic ranges to **3b** and other C6-prenylated indole derivatives[7] and was therefore identified as a C6-alkylated derivative. It seems that the smaller methylallyl group was placed in the reaction site with a similar distance from its C-1′ to C-4 and C-5 in FgaPT2 or to C-5 and C6 in 5-DMATS, so that attacking from both positions was possible.

To test the importance of the double bond at the β-position to diphosphate, we prepared 3-methylbutanyl diphosphate **5** and 4-methyl-3-pentenyl diphosphate **6** (Figure 1) and used them as alkyl donors for FgaPT2, 5-DMATS, and 7-DMATS. As shown in Figure 2, no product formation was detected for any of the enzyme assays. This proved that the double bond at the β-position is essential for the alkylation. Based on the protein structures of several prenyltransferases, it was proposed that a carbocation will be first created with the help of several basic amino acids.[10] This carbocation must be then stabilized by several amino acid residues, e.g. tyrosine, as well as by the aromatic ring system of the substrate itself to protect its reaction with undesired nucleophiles.[10a] Our data in this study showed that stabilization of the carbocation by itself, *via* a β-unsaturated C−C bond, is also essential for a successful Friedel−Crafts alkylation. It would be of great interest to create enzyme derivatives, e.g. by site directed mutagenesis, which can stabilize such ions and accept also saturated alkyl diphosphates as alkyl donors.

To compare the biochemical properties of FgaPT2 and 5-DMATS toward DMAPP and its analogues, kinetic parameters were calculated from Hanes−Woolf and Eadie−Hofstee transformations (Table 1). All the reactions apparently followed Michaelis−Menten kinetics. FgaPT2 accepted its natural substrate DMAPP with a K_M value of 4 μM, much better than MAPP and 2-pen-PP with K_M values at 40 and 56 μM, respectively. In contrast, 5-DMATS has no significant preference and even a higher affinity to MAPP than to DMAPP. The turnover numbers of FgaPT2 and 5-DMATS toward unnatural alkyl diphosphates were found to be less than 3% of those with DMAPP but are still within a realized range for use in chemical reactions. For both enzymes, 2-pen-PP was accepted with a higher reaction velocity than MAPP.

Table 1. Kinetic Parameters of FgaPT2 and 5-DMATS with DMAPP and Analogues as Alkyl Donors

enzyme	donor	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}\cdot M^{-1}$)
FgaPT2	DMAPP[a]	4	0.37	92 500
	MAPP	40	0.009	232
	2-pen-PP	56	0.011	198
5-DMATS	DMAPP[b]	76	1.3	17 105
	MAPP	40	0.005	136
	2-pen-PP	131	0.023	174

[a] The data were adopted from ref 6b. [b] The data were adopted from ref 2a.

In conclusion, the structure of the natural alkylation reagent DMAPP can be modified and used as an alkyl donor for some members of the DMATS superfamily, which were successfully used for the prenylation of diverse aromatic substrates including indoles, hydroxynaphthalenes, flavonoids, and tetracyclic napthacenediones.[1c,2b,2c,4b] One methyl group in DMAPP can be deleted or shifted, but the double bond at the β-position seems to be essential for the stability of the formed carbocation. In comparison to DMAPP, the alkylation of the smaller MAPP is in part retained and in part shifted to one position, i.e. from C-4 to C-5 by FgaPT2 and from C-5 to C-6 by 5-DMATS. With 2-pen-PP as an alkyl donor, the alkylation position was completely shifted for one position. The acceptance of the modified prenyl donors by prenyltransferases expanded significantly the potential use of these enzymes as catalysts in the chemoenzymatic synthesis. It would be interesting to test the acceptance of the DMAPP analogues by other prenyltransferases.

Acknowledgment. We thank Dr. Laufenberg and Lena Ludwig (Philipps-Universität Marburg) for acquiring mass spectra and for the synthesis of DMAPP, respectively. This work was supported in part by Deutsche Forschungsgemeinschaft Grant Li844/4-1 (to S.-M.L.).

Supporting Information Available. Experimental procedures, HR-MS, NMR data and spectra. This material is available free of charge via Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

(9) Position C-5 (δ_C 119.2) is confirmed by connectivities of H-5 (δ_H 6.74) to C-7 (δ_C 109.5) and C-9 (δ_C 124.7) as well as connectivities of H-10 (δ_H 3.29 and 2.90) and H-2 (δ_H 7.16) to C-9.

(10) (a) Metzger, U.; Schall, C.; Zocher, G.; Unsöld, I.; Stec, E.; Li, S.-M.; Heide, L.; Stehle, T. *Proc. Natl. Acad. Sci. U.S.A* **2009**, *106*, 14309–14314. (b) Lik, L. Y. P.; Tanner, M. E. *J. Am. Chem. Soc.* **2009**, *131*, 13932–13933.

Expansion of enzymatic Friedel-Crafts Alkylation on Indoles: Acceptance of unnatural ß-unsaturated Allyl Diphosphates by DMAT Synthases

Supporting Information

Mike Liebhold[†], Xiulan Xie[§] and Shu-Ming Li*[,†]

[†]Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17a, 35037 Marburg, Germany , [§]Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany.

Experimental Procedures

Chemicals

Dimethylallyl diphosphate (DMAPP), methylallyl diphosphate (MAPP) [*trans:cis*= 5:1], 2-*trans*-pentenyl diphosphate (2-*trans*-pen-PP), 3-metylbutanyl diphosphate and 4-methyl-3-pentenyl diphosphate were prepared according to the method described for geranyl diphosphate by Woodside et al..[1] Crotyl chloride (Sigma Aldrich, Taufkirchen, Germany), 1-chloro-3-methylbutane (TCI Europe, Zwijndrecht, Belgium) and 5-bromo-2-methyl-2-penten (Alfa Aesar, Karlruhe, Germany) were used for the synthesis of MAPP, 3-metyl-butanyl and 4-methyl-3-pentenyl diphosphate, respectively. The synthesis of 2-pen-PP started with *trans*-2-pentenol (Sigma Aldrich, Taufkirchen, Germany). L-tryptophan was purchased from Roth (Karlsruhe, Germany).

Overproduction and purification of recombinant Proteins

Protein overproduction and purification was carried out as described previously for FgaPT2[2], 5-DMATS[3] and 7-DMATS, respectively[4].

Enzyme assays for determination of relative activities

The enzymatic reaction mixtures (100 µl) for determination of the relative activities with DMAPP and its analogues contained 1 mM L-tryptophan, 10 mM $CaCl_2$, 2 mM of DMAPP or its analogues, 1.0-1.5% (v/v) glycerol, 2.5% (v/v) dimethyl sulfoxide (DMSO), 50 mM Tris pH 7.5 and 5 µg of purified recombinant protein. The reaction mixtures were incubated at 37 °C for 16 h. The reactions were then terminated with 100 µl MeOH. Protein was removed by centrifugation at 13.000 x g for 10 min.

Enzyme assays for isolation and structure elucidation

Assays for isolation of the enzyme products were carried out in large scales (10 ml-25 ml) containing L-tryptophan (1 mM), alkyl diphosphates (1-2 mM), $CaCl_2$ (10 mM), glycerol (1.0%-1.5% v/v), DMSO (2.5% v/v), Tris HCl (50 mM pH 7.5) and up to 0.25 mg protein per ml assay. For assays with 5-DMATS, protein was added in 2 consecutive steps with duration of 4-6 h. After incubation for 16 h at 37 °C, the reaction mixtures were terminated with the same volume of MeOH as that of the enzyme assay. The assays were centrifuged for 10 min at 6000 rpm to precipitate protein, concentrated on a rotary evaporator to 1-2 ml and used as samples for separation on HPLC.

Enzyme assays for determination of kinetic parameters

Assays for determination of kinetic parameters contained 1 mM L-tryptophan, 10 mM $CaCl_2$, glycerol (1.0-1.5% v/v), 2.5% v/v DMSO, 50 mM Tris HCl pH 7.5 and alkyl diphosphates in final concentrations of 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mM. Protein amount, incubation time and temperature varied between 5 µg and 10 µg, 30 min and 60 min and 30 °C and 37 °C, respectively. The reactions were then terminated with 100 µl MeOH. Protein was removed by centrifugation at 13.000 x g for 10 min.

HPLC analysis and isolation of enzyme products for structure elucidation

The enzyme products were routinely analyzed by HPLC on an Agilent series 1200 by using a Multospher 120 RP-18 column (250 x 4 mm, 5µm C+S Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 ml min^{-1}. Water (solvent A) and methanol (solvent B) were used as solvents for analysis and isolation of the enzyme products. A linear gradient of 40-100% (v/v) solvent B in 15 min to 20 min was used for analysis of the enzyme products. The column was then washed with 100% solvent B for 5 min and equilibrated with 40% solvent B for another 5 min. Detection was carried out by photo diode array detector.

The products of FgaPT2 with MAPP were isolated by repeated chromatography on a Multospher 120 RP18 column (250 x 10 mm, 5 μm) with different gradients, *e.g.* 60-100% in 20 min, at 2.5 ml min^{-1} and the products with 2-pentenyl-PP on a Multospher 120 RP18 column (250 x 4 mm, 5 μm) by using a gradient of 50-80% in 15 min at a flow rate of 1 ml min^{-1}. For isolation of 5-DMATS products, a gradient of 40-100% in 15min with a flow rate of 1ml min^{-1} was used. After each run, the columns were then washed with 100% solvent B for 5 min followed by 5min of equilibration with the corresponding condition at the beginning of each run.

NMR and MS analyses

^{1}H and ^{31}P NMR spectra were recorded on a JEOL ECA-500 or a Bruker Avance 500 MHz spectrometer. ^{13}C and two-dimensional spectra HSQC and HMBC were recorded on a Bruker Avance 500 MHz spectrometer. Chemical shifts were referenced to the signal of DMSO-d_6 at 2.50 ppm or CD_3OD at 3.30 ppm. Spectra were processed with MestRENova 5.2.2. The isolated compounds were also analyzed by electron impact mass spectrometry (EI-MS) or electrospray ionization mass spectrometry (ESI-MS) on an Auto SPEC (Micromass Co. UK Ltd.).

Reference List

(1) Woodside, A. B.; Huang, Z.; Poulter, C. D. *Org. Synth.* **1988**, *66*, 211.

(2) Steffan, N.; Unsöld, I. A.; Li, S.-M. *Chembiochem* **2007**, *8* , 1298.

(3) Yu, X.; Liu, Y.; Xie, X.; Zheng, X.-D.; Li, S.-M. *J. Biol. Chem.* **2012**, *287* , 1371.

(4) Kremer, A.; Westrich, L.; Li, S.-M. *Microbiology* **2007**, *153* , 3409.

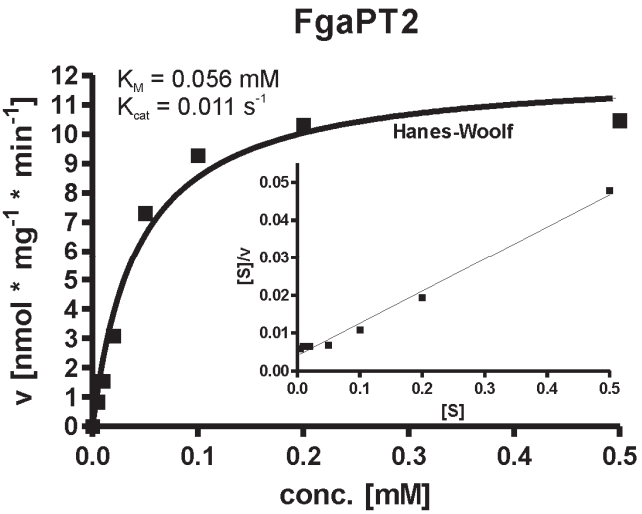


Figure S1. Dependence of the product formation of the FgaPT2 reaction on the presence of 2-pen-PP.

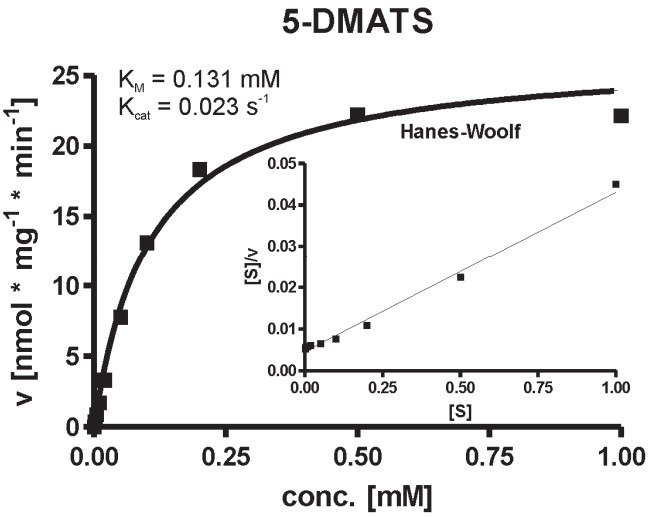


Figure S2. Dependence of the product formation of the 5-DMATS reaction on the presence of 2-pen-PP.

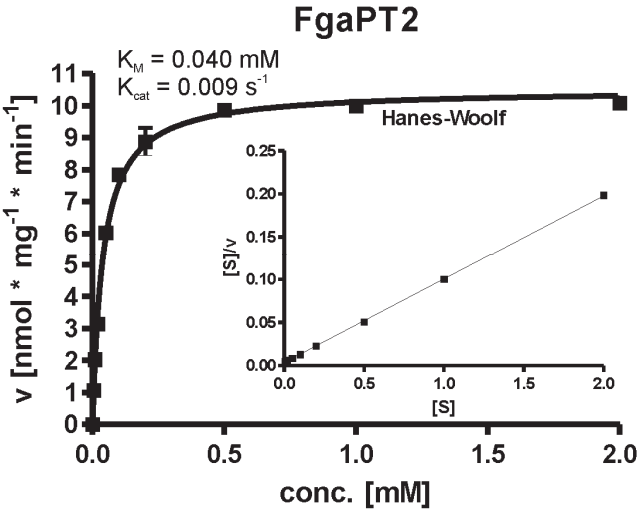


Figure S3. Dependence of the product formation of the FgaPT2 reaction on the presence of MAPP.

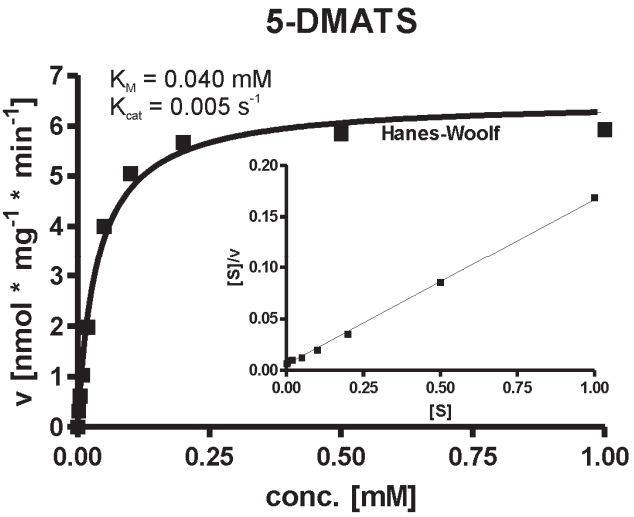


Figure S4. Dependence of the product formation of the 5-DMATS reaction on the presence of MAPP.

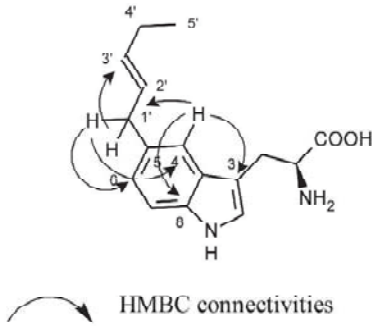


Figure S5. HMBC connectivities of 5-(2-*trans*-pentenyl)-L-tryptophan (**3a**).

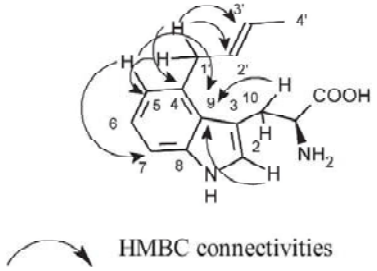


Figure S6. HMBC connectivities of 4-(2-*trans*-butenyl)-L-tryptophan (**4a**).

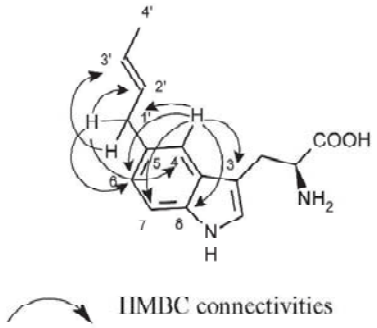
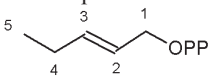
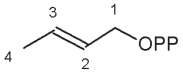
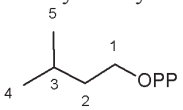
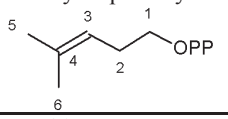


Figure S7. HMBC connectivities of 5-(2-*trans*-butenyl)-L-tryptophan (**4b**).

Table S1. HR-EI-MS and HR-ESI-MS data.

comp.	chem. formula	mode	calculated	measured	deviation (ppm)
3a	$C_{16}H_{20}N_2O_2$	EI	272.1525 $[M]^+$	272.1518 $[M]^+$	2.6
3b	$C_{16}H_{20}N_2O_2$	EI	272.1525 $[M]^+$	272.1509 $[M]^+$	5.7
4a	$C_{15}H_{18}N_2O_2$	ESI	259.1447 $[M+H]^+$	259.1471 $[M+H]^+$	-9.6
4b	$C_{15}H_{18}N_2O_2$	ESI	259.1447 $[M+H]^+$	259.1434$[M+H]^+$	4.8
4b + 4c	$C_{15}H_{18}N_2O_2$	EI	258.1368 $[M]^+$	258.1388 $[M]^+$	-7.4

Table S2. ^{1}H NMR Data of alkyl diphosphates.

	2-pen-PP 	MAPP 	3-methylbutanyl-PP 	4-methyl-3-pentenyl-PP 
pos.	δ_H, multi, *J*	δ_H, multi, *J*	δ_H, multi, *J*	δ_H, multi, *J*
1	4.48, d, 6.6 4.47, d, 7.5	4.43, t, 6.7	3.94, q, 6.8	3.97, q, 7.0
2	6.00, dt, 15.5, 6.3	5.75, dt, 15.3, 6.4	1.50, q, 6.9	2.43, q, 7.0
3	5.74, dt, 15.5, 6.3	5.93, dq, 15.3, 6.6	1.67, m	5.30, m
4	2.15, m	1.77, dd, 6.6 0.9	0.87, d, 6.7	-
5	1.06, t, 7.5	-	0.87, d, 6.7	1.73, s[a]
6	-	-	-	1.79, s[a]

[a]: assignments of signals are interchangeable.

Table S2. ^{31}P NMR Data of alkyl diphosphates.

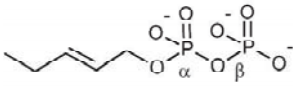
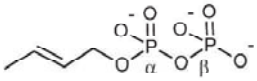
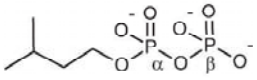
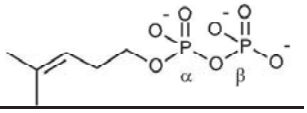
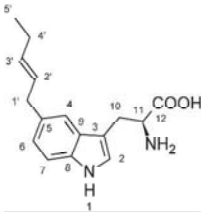
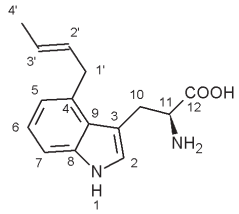
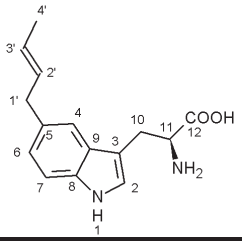
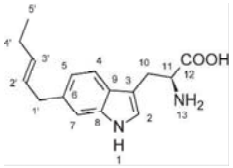
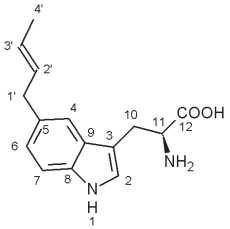
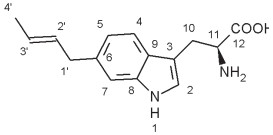
	2-pen-PP 	MAPP 	3-methylbutanyl-PP 	4-methyl-3-pentenyl-PP 
	δ_P, multi, *J*	δ_P, multi, *J*	δ_P, multi, *J*	δ_P, multi, *J*
P_α	-10.05, d, 20.9	-9.98, d, 20.9	-9.87, d, 20.9	-9.91, d, 20.9
P_β	-9.37, d, 20.9	-8.49, d, 20.9	-9.29, d, 20.9	-8.45, d, 20.9

Table S4. ^{1}H NMR Data of FgaPT2 and 5-DMATS products with DMAPP analogues

enzyme	FgaPT2						5-DMATS		
comp	**3a** 		**4a** 		**4b** 		**3b** 	**4b**[#] 	**4c**[#] 
Pos.	$\delta_{H,}$ *multi, J*	δ_C	$\delta_{H,}$ *multi, J*	δ_C	$\delta_{H,}$ *multi, J*	δ_C	$\delta_{H,}$ *multi, J*	$\delta_{H,}$ *multi, J*	$\delta_{H,}$ *multi, J*
1	-	-	-	-	-	-	-	-	-
2	7.16, d, 1.9	124.0	7.17, br. s	124.3	7.15, s	125.3	7.11, s	7.14, br s	7.11, s
3	-	109.2	-	110.5	-	109.2	-	-	-
4	7.32, s	117.2	-	132.7	7.48, s	118.6	7.59, dd, 8.2, 0.6	7.49, dd, 1.4, 0.6	7.59, dd, 8.1, 0.6
5	-	130.2	6.74, d, 7.1	119.2	-	133.0	6.89, dd, 8.2, 1.5	-	6.88, dd, 8.2, 1.5
6	6.90, dd, 8.3, 1.3	121.8	6.97, t, 7.6	121.0	6.95, dd, 8.3, 1.4	123.9	-	6.95, dd, 8.3, 1.4	-
7	7.26, d, 8.3	111.0	7.20, dd, 8.1, 0.6	109.5	7.27, d, 8.3	112.3	7.15, dd, 1.5, 0.6	7.26, dd, 8.3, 0.6	7.14, br. s
8	-	134.9	-	137.2	-	137.1	-	-	-
9	-	127.2	-	124.7	-	128.6	-	-	-
10	3.29 [§] 2.90, dd, 15.1, 9.5	27.0	3.58, dd. 15.5, 2.8 2.90, dd, 15.5, 10.8	29.1	3.50, dd, 15.2, 3.8 3.11, dd, 15.2, 9.6	28.5	3.47, ddd, 15.3, 4.0, 0.9 3.10, dd, 15.3, 9.3	3.48,ddd, 15.2, 4.1, 0.9 [b] 3.09, dd, 15.2, 9.7 [c]	3.46,ddd, 15.2, 4.1, 0.9 [b] 3.07, dd, 15.2, 9.7 [c]
11	3.43, dd, 9.5, 4.1	54.5	3.37, dd, 10.8, 2.8	55.6	3.87, dd, 9.6, 3.8	56.6	3.82, dd, 9.3, 4.0	3.82, dd, 9.7, 4.1 [d]	3.81, dd, 9.7, 4.1 [d]
12	10.78, s (COOH)	169.7	-	169.6	-	169.4	-	-	-
1′	3.36, d, 5.5	38.5	3.75, dd, 15.4, 6.3 3.63, dd, 15.4, 6.3	35.7	3.39, d, 6.6	40.3	3.37, d, 6.2	3.38, d, 6.9 [e]	3.37, d, 6.9 [e]
2′	5.57, m [a]	129.3	5.64, dtd, 15.2, 6.4, 1.6	131.2	5.63, dtq, 15.0, 6.7, 1.5	132.9	5.57, m [b]	5.62, dtq, 15.2, 6.4, 1.5 [f]	5.61, dtq, 15.2, 6.4, 1.5 [f]
3′	5.53, m [a]	131.6	5.47, dq, 15.2, 6.5	125.0	5.50, dqt, 15.0, 6.3, 1.2	126.0	5.53, m [b]	5.50, dqt, 15.2, 6.4, 1.4 [g]	5.49, dqt, 15.2, 6.4, 1.4 [g]
4′	2.00, m	24.7	1.62, d, 6.3	17.7	1.66, dd, 6.3, 1.3	18,0	2.03, m	1.66, dd, 6.2, 1.2 [h]	1.65, dd, 6.3, 1.3 [h]
5′	0.94, t, 7.4	13.6	-	-	-	-	0.98, t, 7.5	-	-
solvent	2.50 (DMSO-d_6)	39.5	2.50 (DMSO-d_6)	39.5	3.30 CD_3OD)	49.0	3.30 (CD_3OD)	3.30 (CD_3OD)	3.30 (CD_3OD)

[§] signals overlapping with those of solvent; [#]**4b+4c** were isolated as mixture from enzyme assay of 5-DMATS; [a,b,c,d,e,f,g,h]: assignments of signals with the same letter are interchangeable.

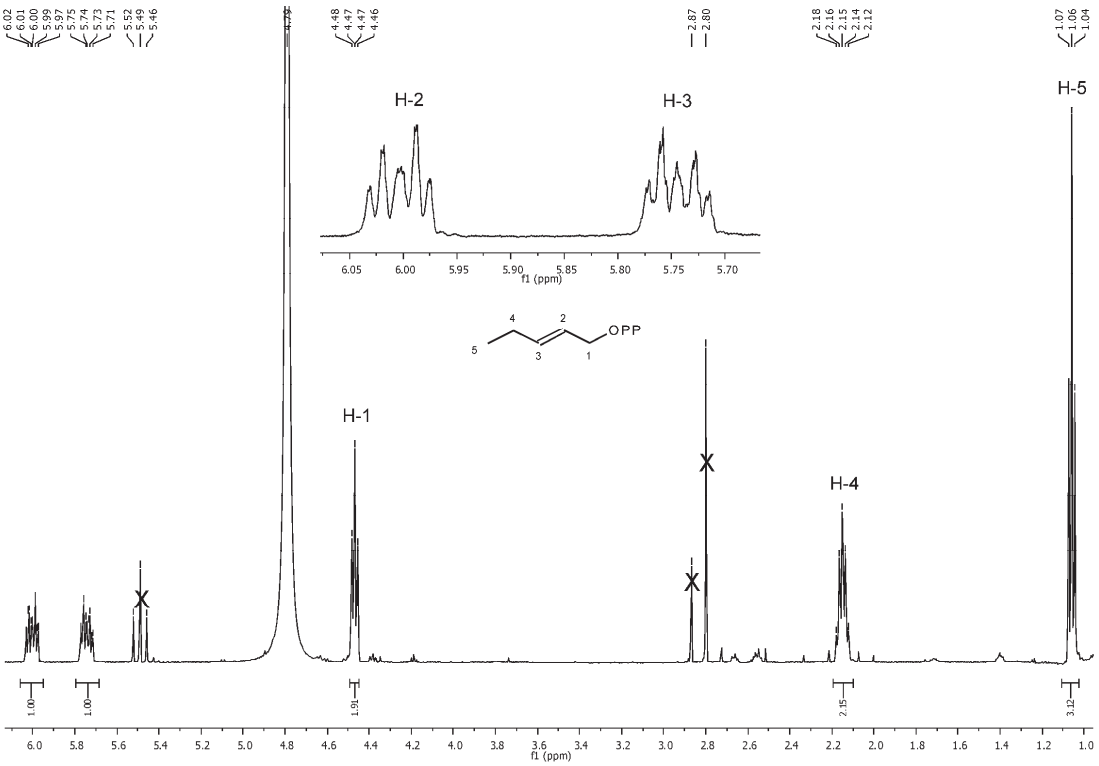


Figure S8. ^{1}H NMR spectrum of 2-pentenyl diphosphate in D_2O.

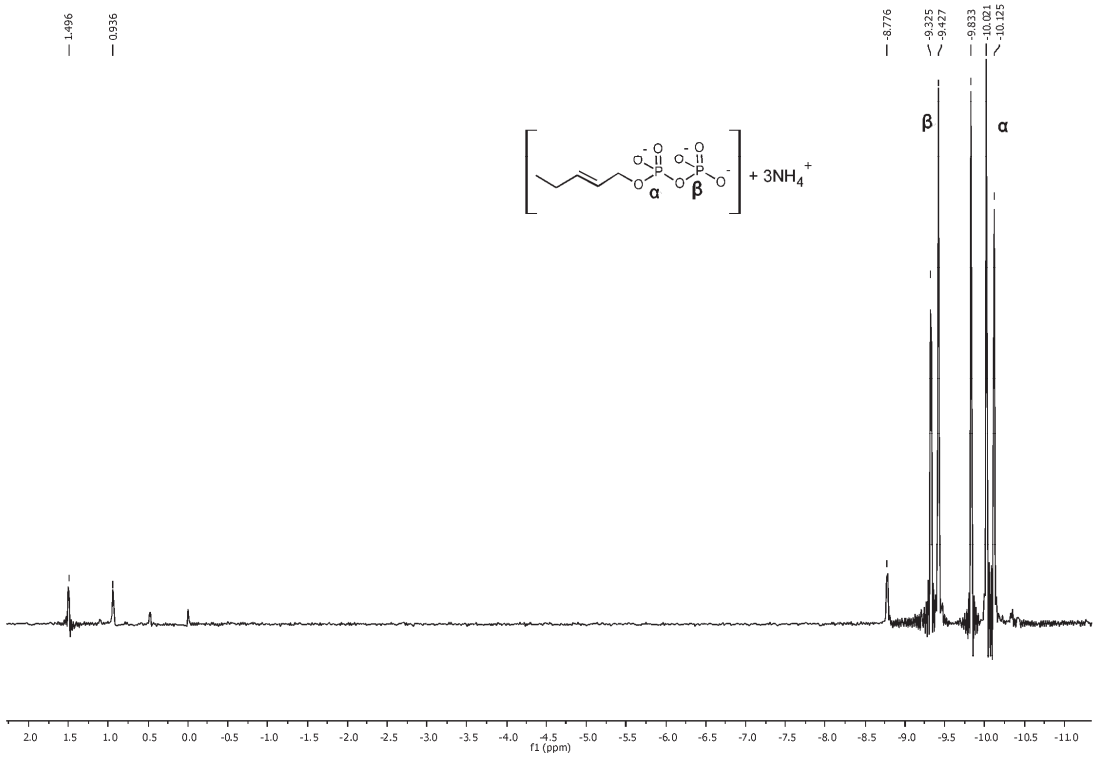


Figure S9. ^{31}P NMR spectrum of 2-pentenyl diphosphate in D_2O.

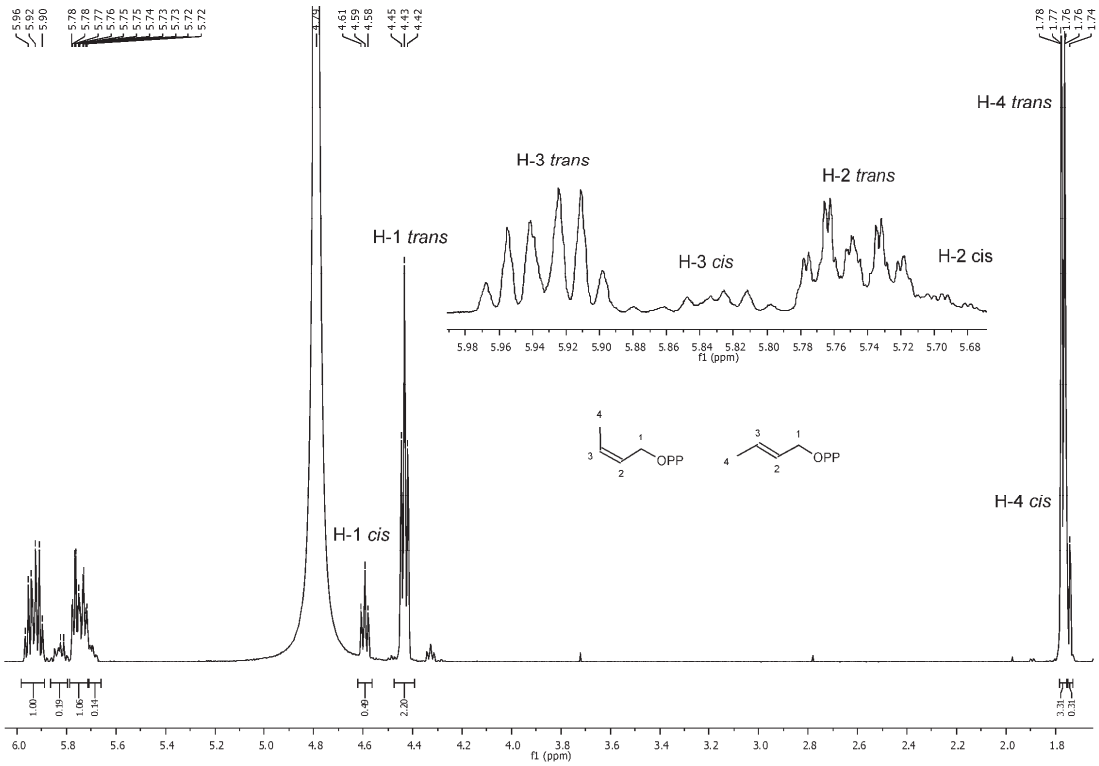


Figure S10. ^{1}H NMR spectrum of MAPP in D_2O.

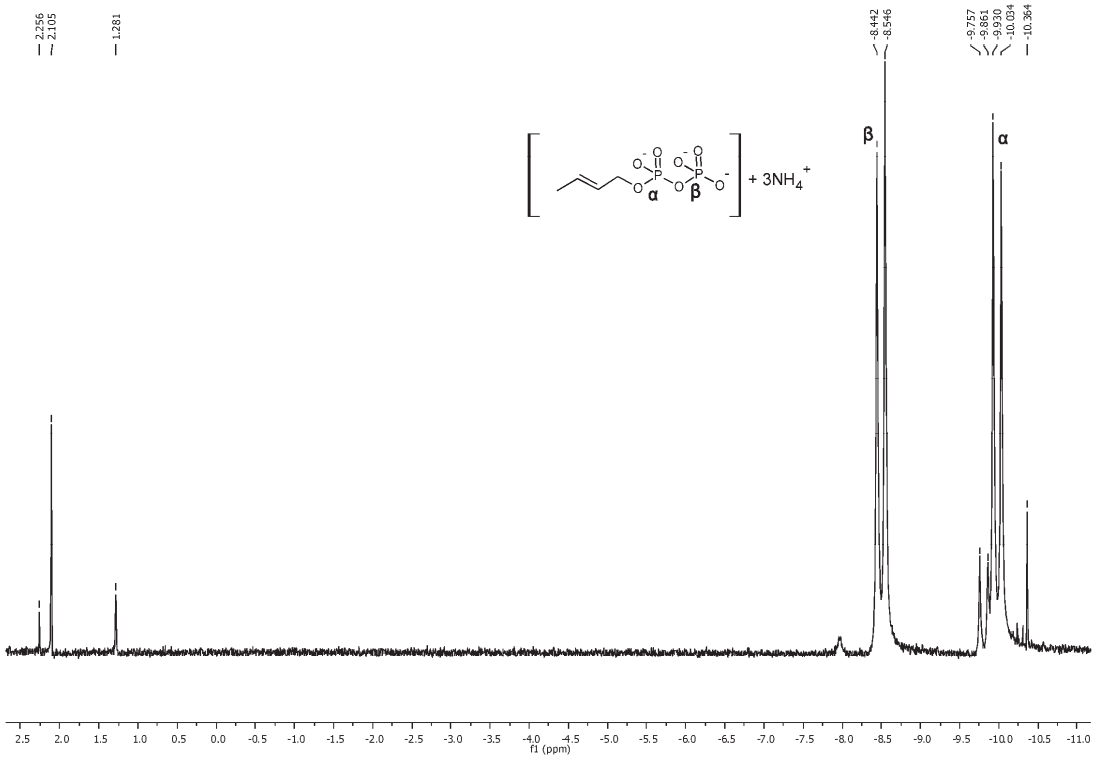


Figure S11. ^{31}P NMR spectrum of MAPP in D_2O.

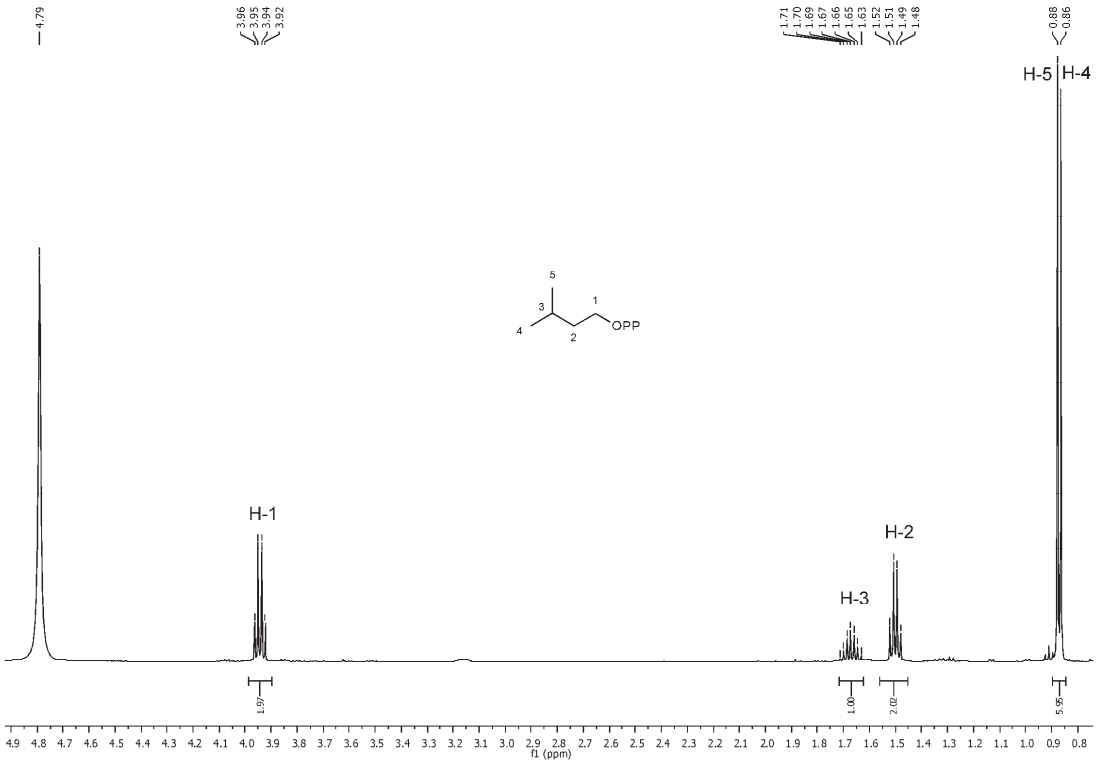


Figure S12. ^{1}H NMR spectrum of 3-methyl-butanyl-PP in D_2O.

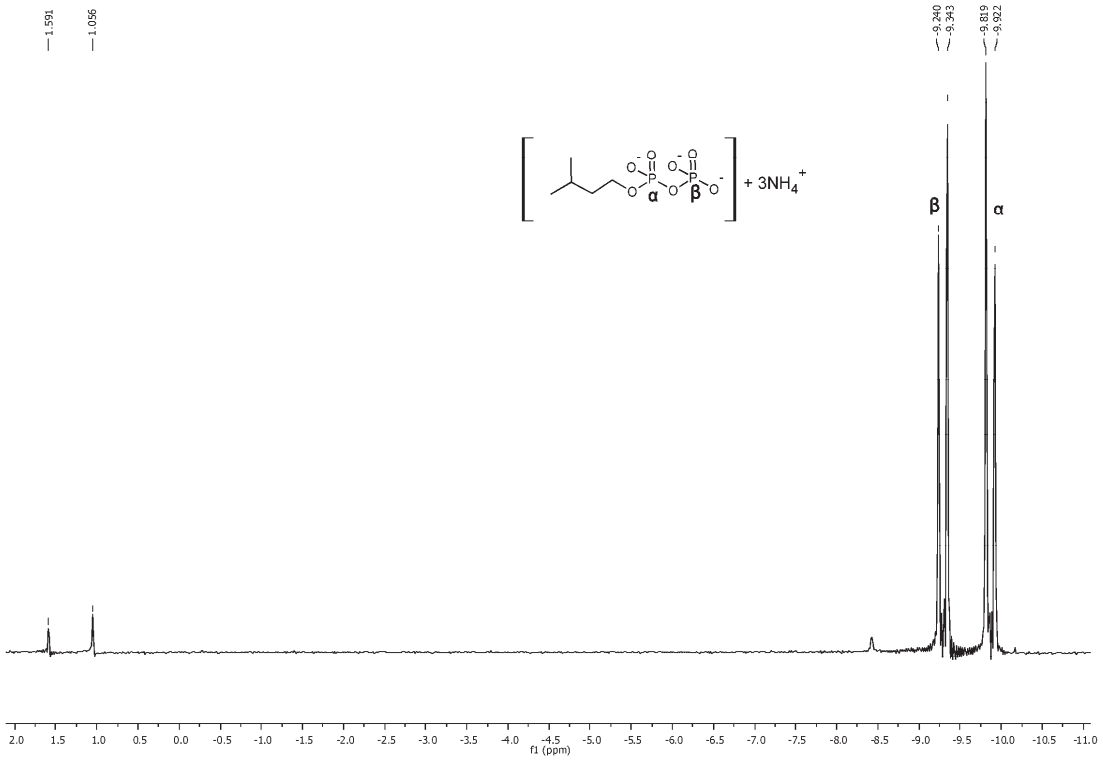


Figure S13. ^{31}P NMR spectrum of 3-methylbutanyl diphosphate in D_2O.

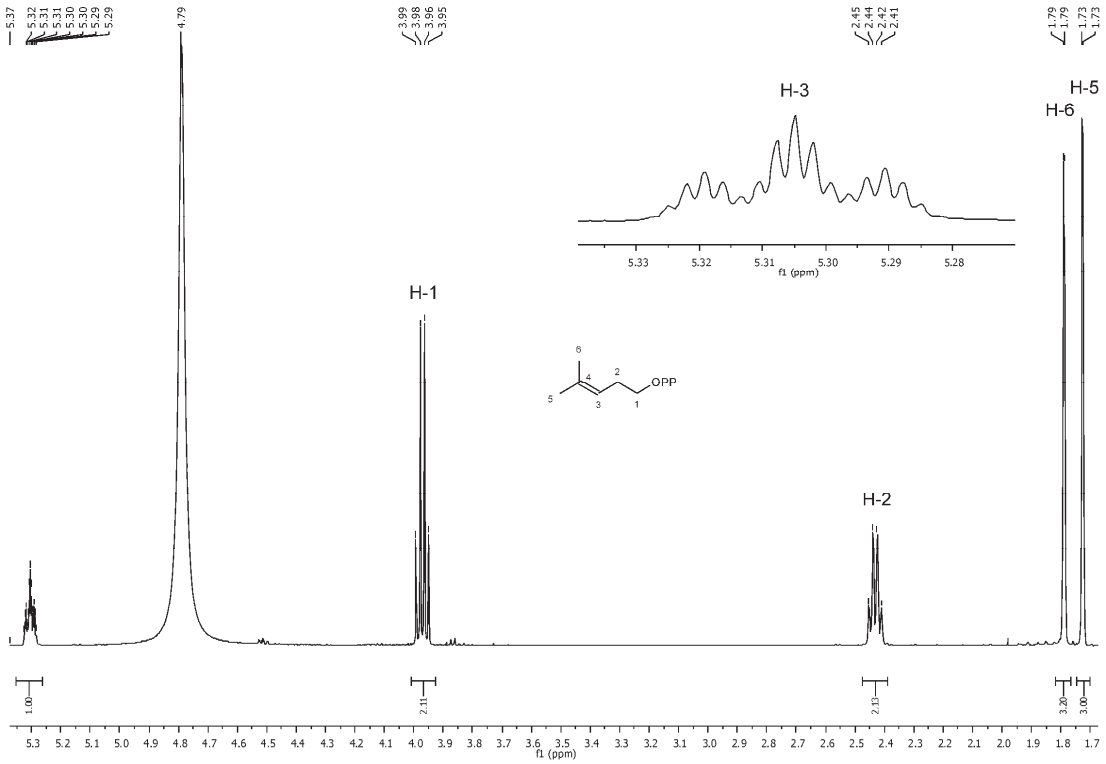


Figure S14. ^{1}H NMR spectrum of 4-methyl-3-pentenyl diphosphate in D_2O.

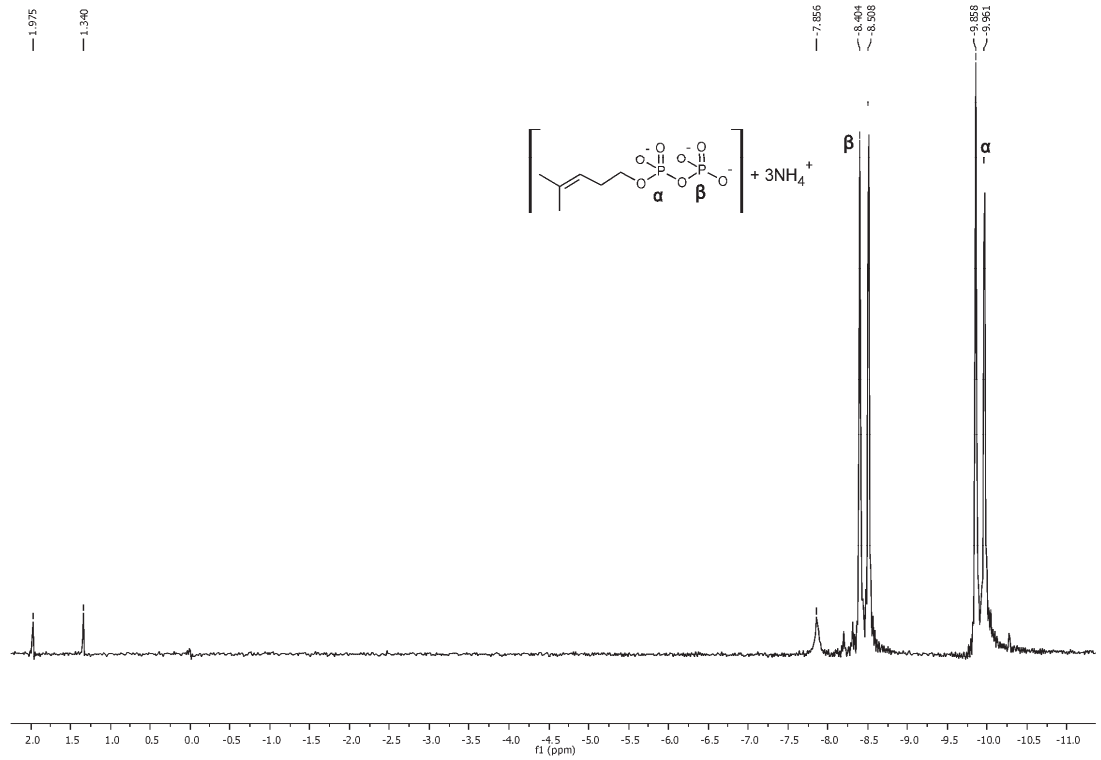


Figure S15. ^{31}P NMR spectrum of 4-methyl-3-pentenyl diphosphate in D_2O.

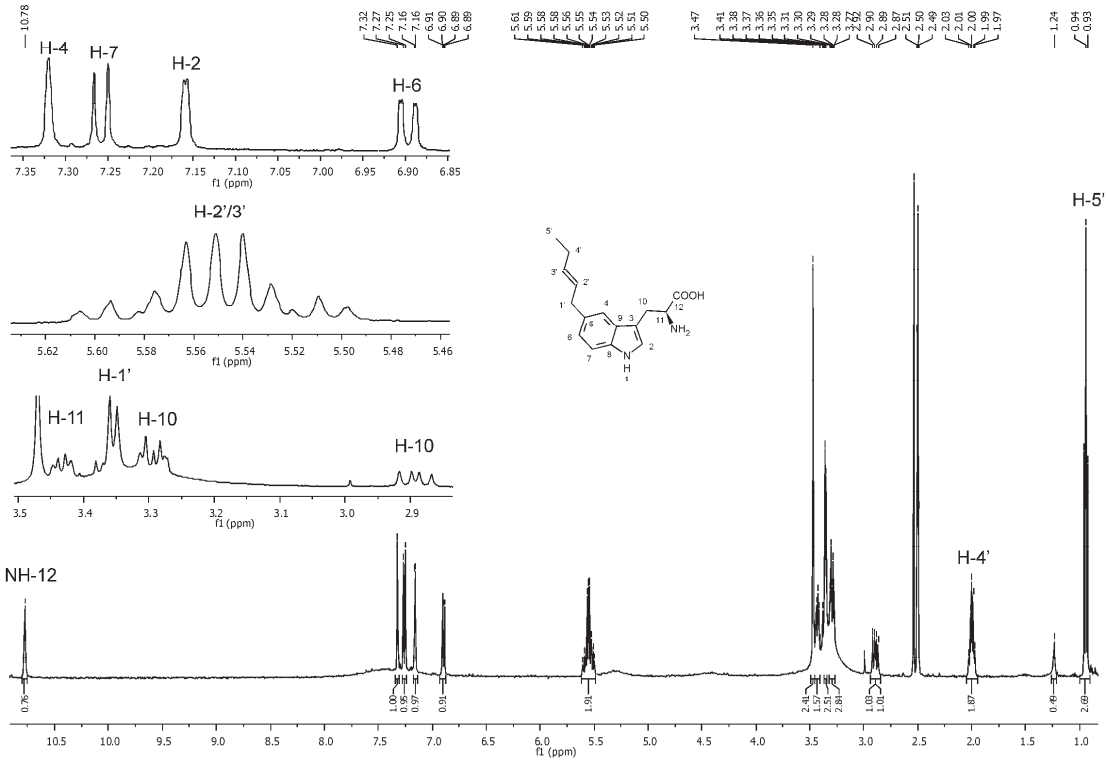


Figure S16. [1]H NMR spectrum of 5-(2-*trans*-pentenyl)-L-tryptophan (**3a**) in DMSO-d_6.

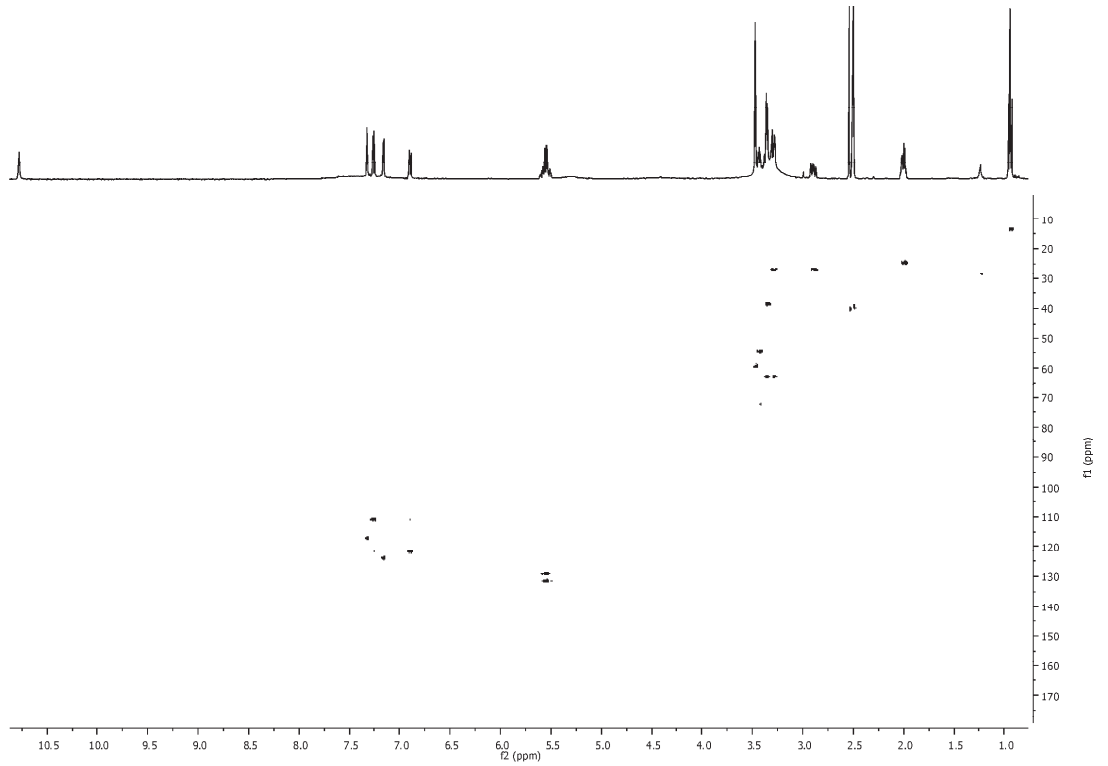


Figure S17. HSQC spectrum of 5-(2-*trans*-pentenyl)-L-tryptophan (**3a**) in DMSO-d_6.

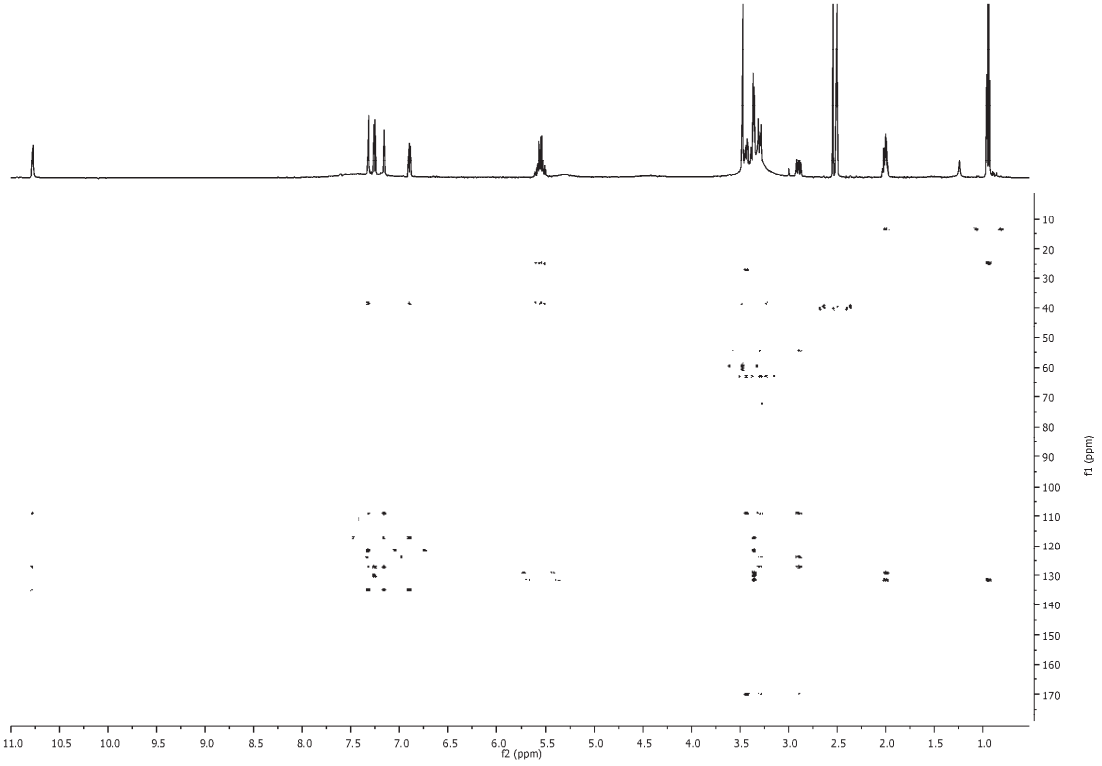


Figure S18. HMBC spectrum of 5-(2-*trans*-pentenyl)-L-tryptophan (**3a**) in DMSO-d_6.

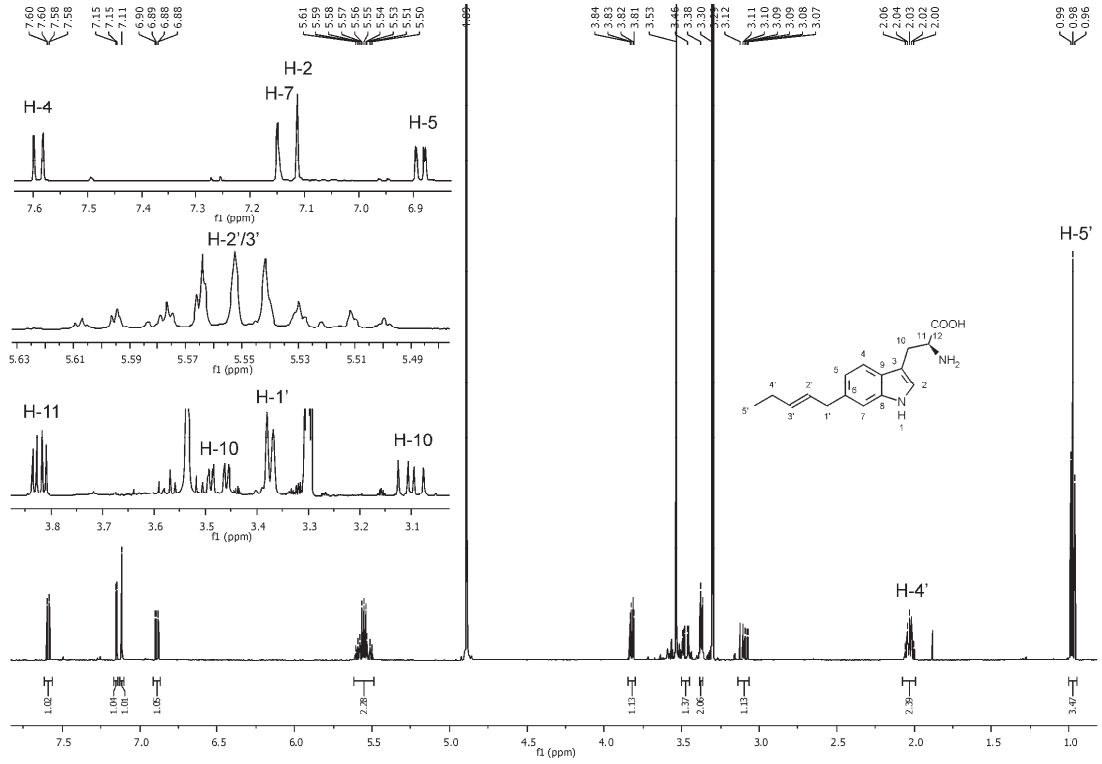


Figure S19. ^{1}H NMR spectrum of 6-(2-*trans*-pentenyl)-L-tryptophan (**3b**) in CD_3OD.

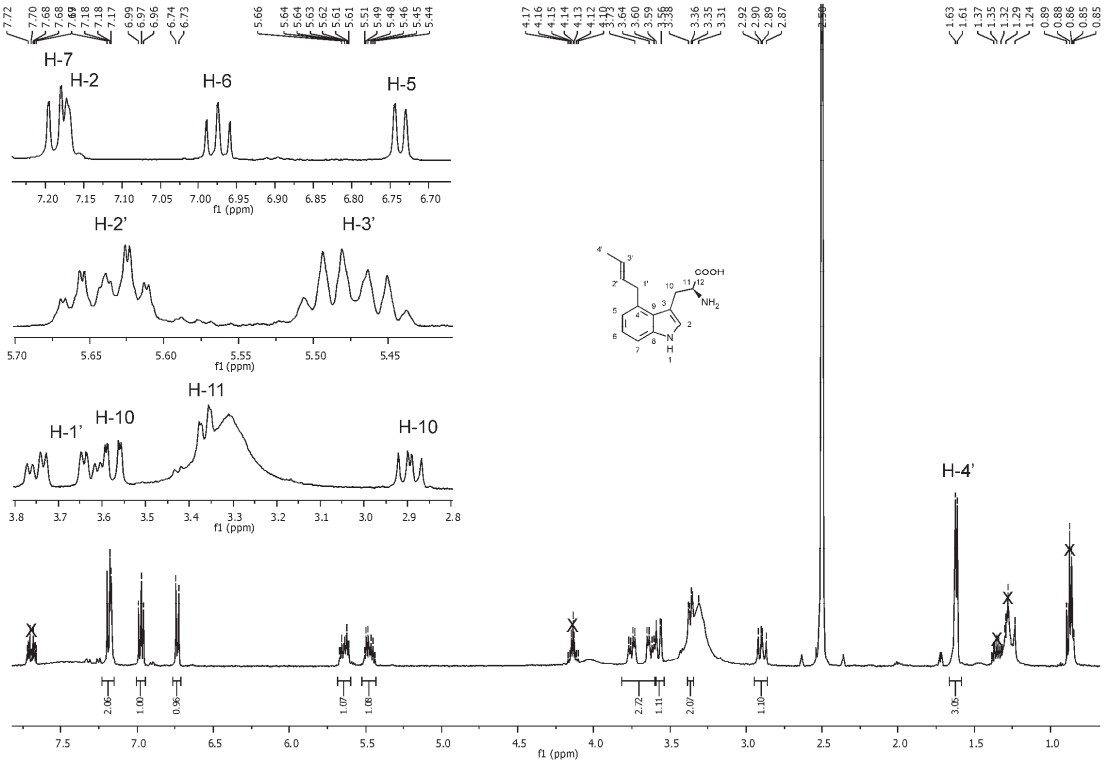


Figure S20. ^{1}H NMR spectrum of 4-(2-*trans*-butenyl)-L-tryptophan (**4a**) in DMSO-d_6.

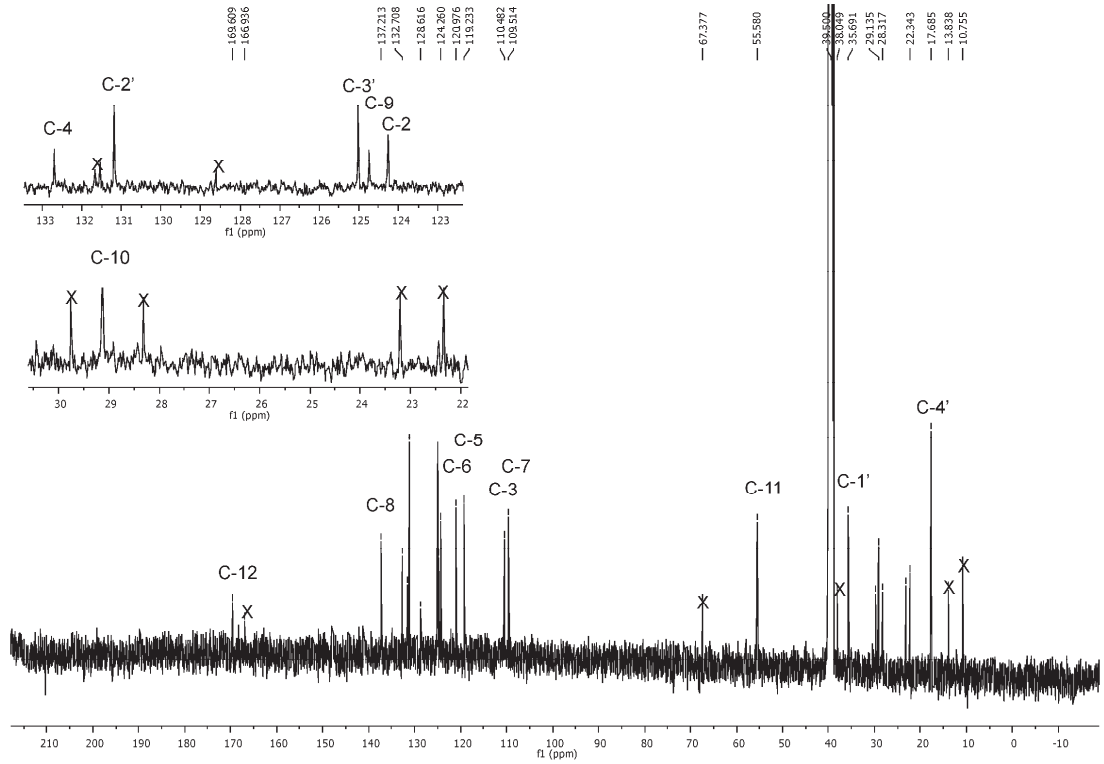


Figure S21. ^{13}C NMR spectrum of 4-(2-*trans*-butenyl)-L-tryptophan (**4a**) in DMSO-d_6.

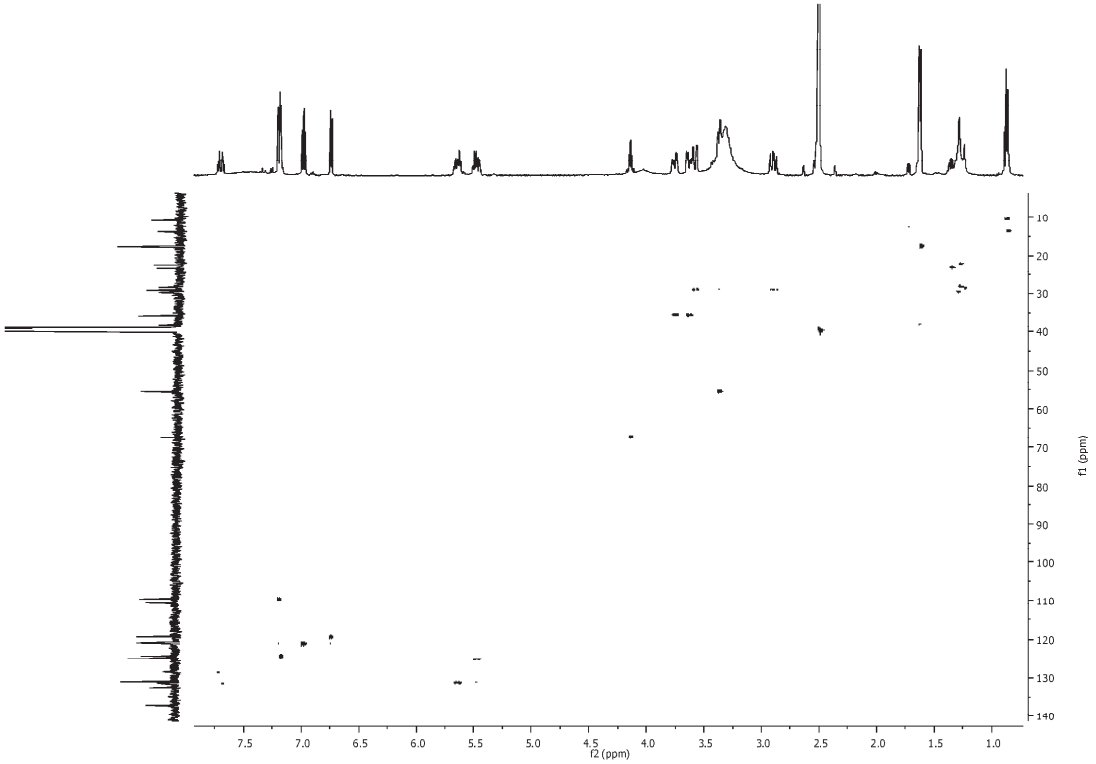


Figure S22. HSQC spectrum of 4-(2-*trans*-butenyl)-L-tryptophan (**4a**) in DMSO-d_6.

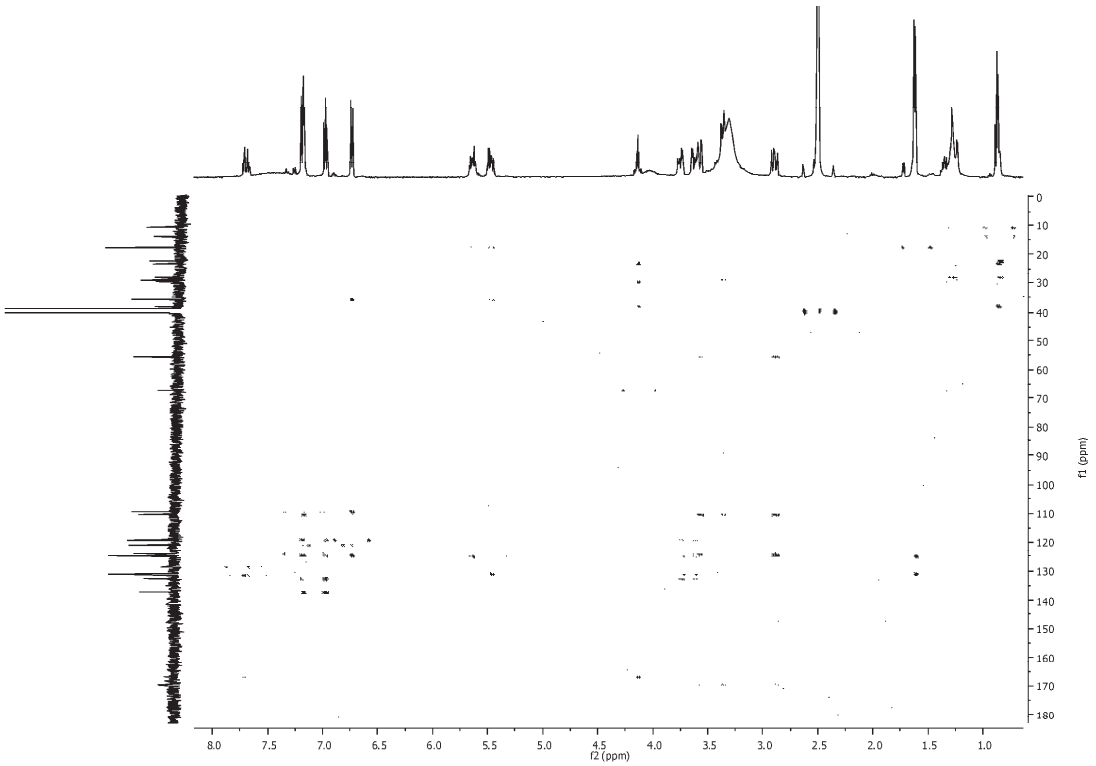


Figure S23. HMBC spectrum of 4-(2-*trans*-butenyl)-L-tryptophan (**4a**) in DMSO-d_6.

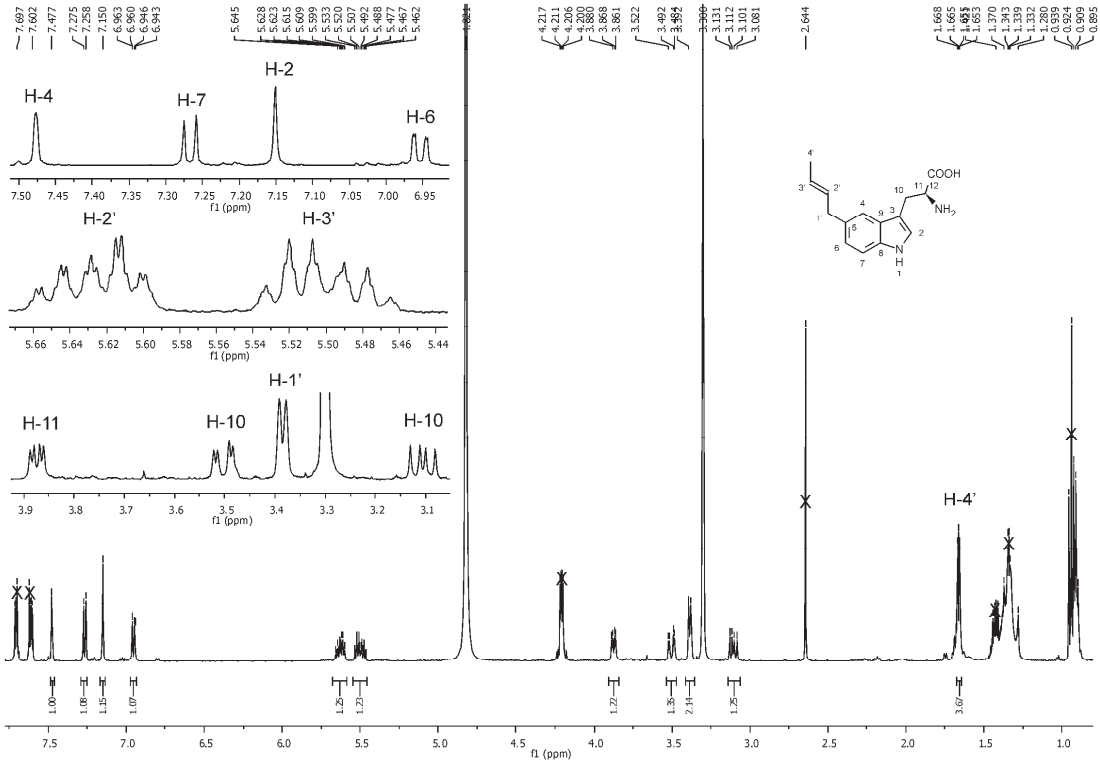


Figure S24. ^{1}H NMR spectrum of 5-(2-*trans*-butenyl)-L-tryptophan (**4b**) in CD_3OD.

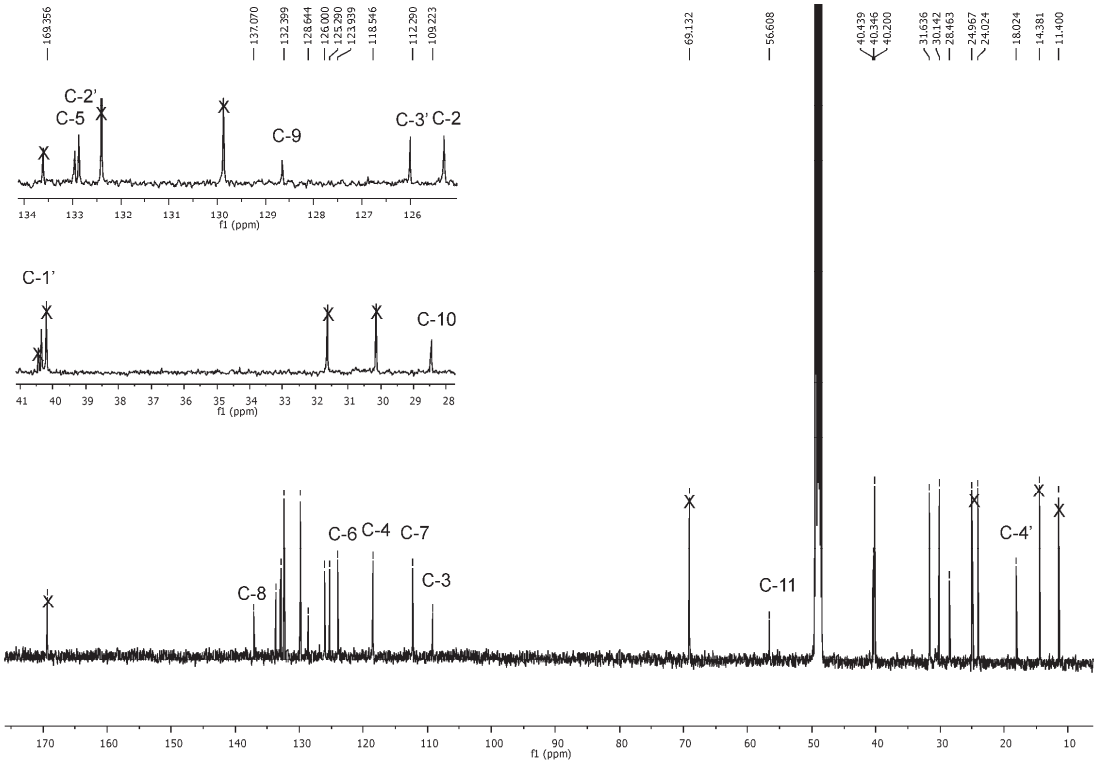


Figure S25. ^{13}C NMR spectrum of 5-(2-*trans*-butenyl)-L-tryptophan (**4b**) in CD_3OD.

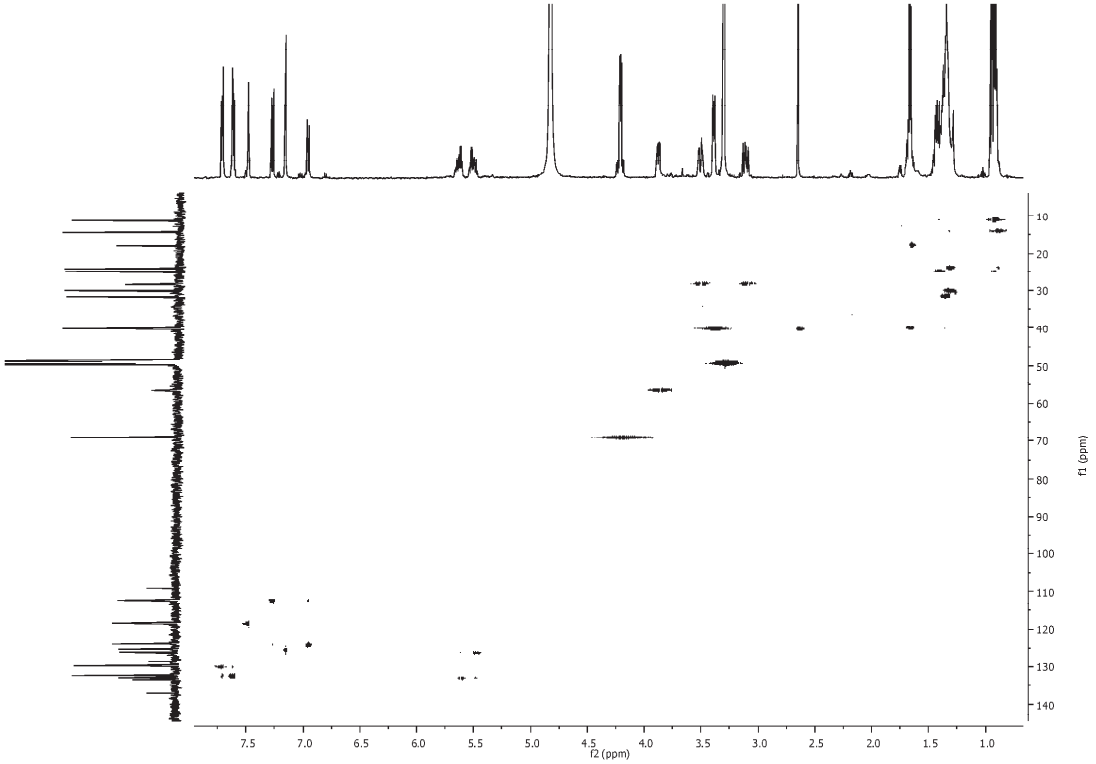


Figure S26. HSQC spectrum of 5-(2-*trans*-butenyl)-L-tryptophan (**4b**) in CD_3OD.

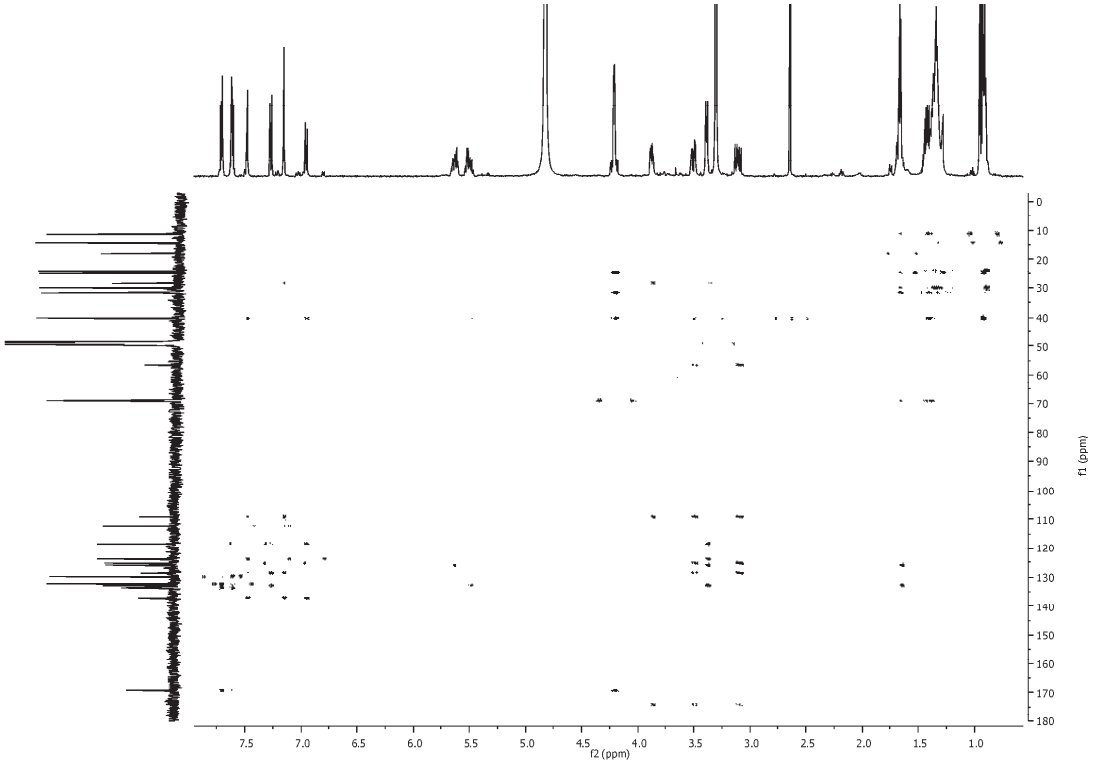


Figure S27. HMBC spectrum of 5-(2-*trans*-butenyl)-L-tryptophan (**4b**) in CD_3OD.

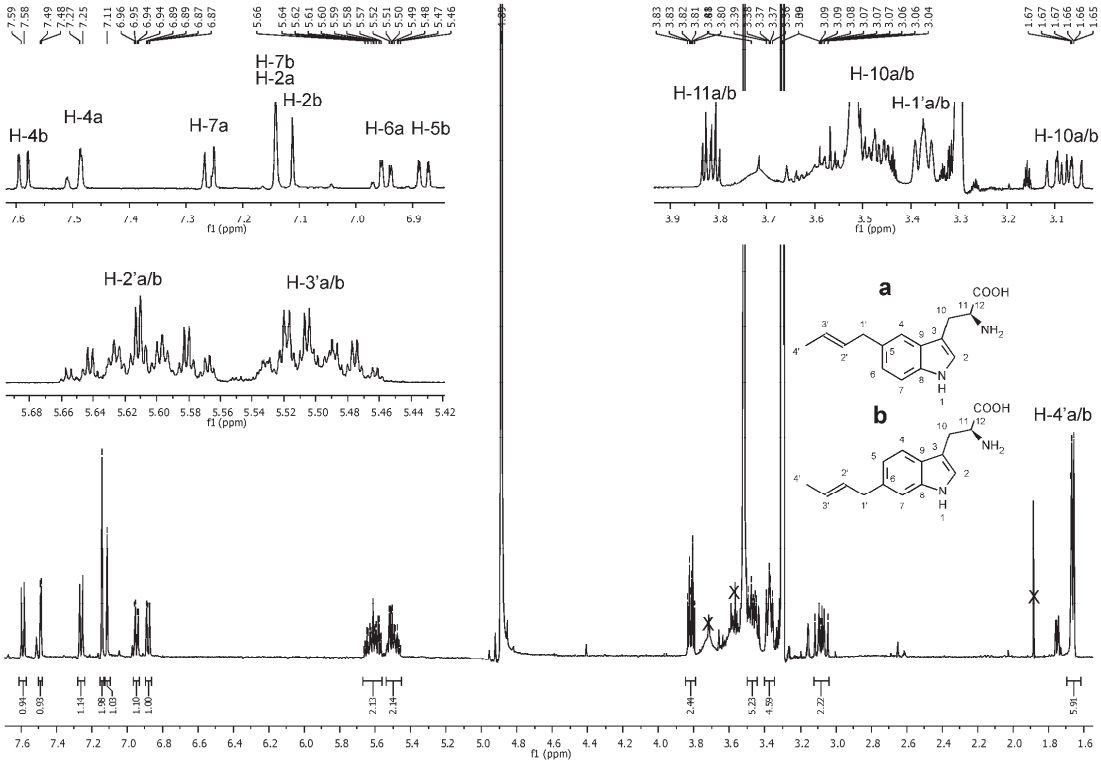


Figure S28. ^{1}H NMR spectrum of mixture 5-(2-*trans*-butenyl)-L-tryptophan + 6-(2-*trans*-butenyl)-L-tryptophan (**4b**+**4c**) in CD_3OD.

4.2. Breaking Cyclic Dipeptide Prenyltransferase Regioselectivity by Unnatural Alkyl Donors

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Breaking Cyclic Dipeptide Prenyltransferase Regioselectivity by Unnatural Alkyl Donors

Mike Liebhold,[†] Xiulan Xie,[§] and Shu-Ming Li*[,†]

Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17a, 35037 Marburg, Germany, and Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany

shuming.li@staff.uni-marburg.de

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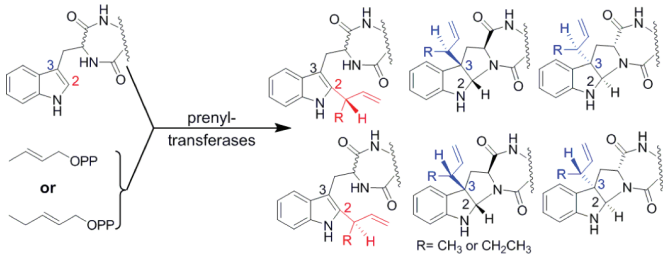
ABSTRACT



The behavior of five cyclic dipeptide prenyltransferases, responsible for C2-regular, C2-reverse, or C3-reverse prenylation, was investigated in the presence of the unnatural alkyl donors monomethylallyl and 2-pentenyl diphosphate. Both substrates were well accepted by the tested enzymes. Interestingly, C2-reverse and C3-reverse monoalkylated derivatives were identified as enzyme products in all of the enzyme assays. These findings indicate their similar reaction characteristics in the presence of unnatural alkyl donors.

Enzymes of the dimethylallyl tryptophan synthase (DMATS) superfamily represent a versatile class of aromatic prenyltransferases. They are involved in the biosynthesis of biologically active natural products in fungi and can be used as biocatalysts for chemoenzymatic synthesis.[1] A large number of these prenyltransferases usually catalyze transfer reactions of a dimethylallyl moiety from dimethylallyl diphosphate (DMAPP) onto tryptophan or tryptophan-containing dipeptides regiospecifically at one of the seven positions of the indole moiety.[1a,c] Furthermore, as in the case of FtmPT1, prenylation can occur in a regular or reverse manner, as for other enzymes illustrated in Scheme 1.[2] Most of these enzymes show significant flexibility for their aromatic substrates but a strict substrate specificity toward their prenyl donors. They usually accept solely DMAPP but not geranyl diphosphate (GPP).[1a,c,2] Only one known enzyme from the DMATS superfamily, i.e., VrtC, utilizes GPP but not DMAPP as prenyl donor.[3] We have recently demonstrated that two L-tryptophan prenyltransferases, FgaPT2 and 5-DMATS, are able to use the unnatural alkyl donors monomethylallyl diphosphate (MAPP, **1**, Figure 1) and 2-pentenyl diphosphate (2-pen-PP, **2**, Figure 1), which contain a double bond in the β-position to pyrophosphate (PPi).[4] Partial or total shift of the alkylation positions was observed in that study. The acceptance of **1** and **2** by FgaPT2 and 5-DMATS as well as alkylation at different positions significantly expands the potential uses of these enzymes as biocatalysts for chemoenzymatic synthesis. The results obtained in that study encouraged us to learn more about the acceptance of these compounds by other

[†] Institut für Pharmazeutische Biologie und Biotechnologie.
[§] Fachbereich Chemie.

(1) (a) Li, S.-M. *Nat. Prod. Rep.* **2010**, *27*, 57–78. (b) Bonitz, T.; Alva, V.; Saleh, O.; Lupas, A. N.; Heide, L. *PLoS One* **2011**, *6*, No. e27336. (c) Yu, X.; Li, S.-M. *Methods Enzymol.* **2012**, *516*, 259–278.

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members of the DMATS superfamily, e.g., by cyclic dipeptide prenyltransferases.

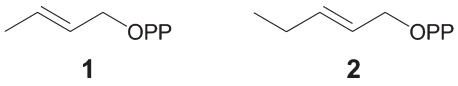


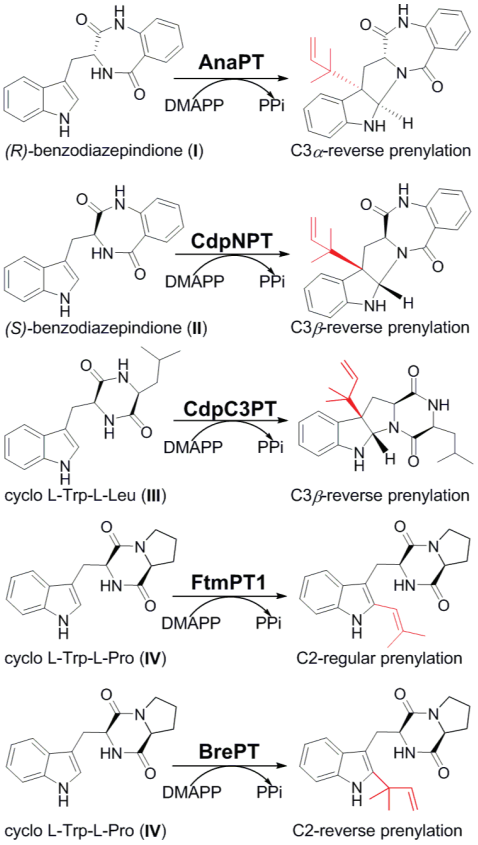
Figure 1. Unnatural DMAPP analogues used as alkyl donors.

For this purpose, we first chose the three C3-prenyltransferases, AnaPT, CdpNPT, and CdpC3PT (Scheme 1), and tested the acceptance of **1** and **2** in the presence of their natural or best accepted aromatic substrates, i.e., (*R*)-benzodiazepindione (**I**), (*S*)-benzodiazepindione (**II**), and *cyclo*-L-Trp-L-Leu (**III**), respectively.[2b−d] HPLC chromatography on both silical gel and reverse-phase C-18 (RP18) columns of the incubation mixtures with 20 μg (AnaPT) or 50 μg of protein (CdpNPT and CdpC3PT) for 16 h showed that both alkyl donors were well accepted by the three tested enzymes. Up to six product peaks, with two to three dominant ones, were detected in the HPLC chromatograms. Total product yields from 32.3% to 98.8% and from 35.5% to 96.5% were calculated for **1** and **2**, respectively. For structure elucidation, we carried out large-scale enzyme incubations and isolated the enzyme products by repeated chromatography, if necessary, on HPLC with RP18 and silical gel columns. The structures of the purified products were elucidated by NMR and MS analyses. For better understanding and comparison, we named the enzyme products by using the number of aromatic substrate (**I**, **II**, or **III**) and alkyl donor (**1** or **2**) as well as the alkylation positions (**a** and **b** for C2- and **c** and **d** for C3-alkylated products). The letters **a** and **b** as well as **c** and **d** denote also the different configurations at C3′ of the alkyl moieties.

In the presence of the natural prenyl donor DMAPP, AnaPT catalyzes α-prenylation at C3 of **I** and other tryptophan-containing cyclic dipeptides.[2b,5] In contrast, CdpNPT and CdpC3PT catalyze β-prenylations at the same position of these compounds.[2c,d]

From incubation mixtures of AnaPT with **I** and **1**, three products, **I-1a**, **I-1c**, and **I-1d**, were isolated and determined as C2-reversely and C3-reversely alkylated derivatives. Both **I-1c** and **I-1d** have an α-alkylation at C3, i.e., *syn-cis* configuration of the fused indoline−diketopiperazine system, as in the case of the product with DMAPP. They differ from each other by their configurations at C3′ of the alkyl moiety, which were determined by HSQC, HMBC, and NOESY experiments. Due to the free rotation of multiple C−C bonds, the configuration at C3′ in **I-1a** cannot be determined in this study. Interestingly, the C2-alkylated **I-1a** was the dominant enzyme product (65.2%) of the C3-prenyltransferase AnaPT, at nearly 2-fold the combined yield of **I-1c** (14.4%) and **I-1d** (19.4%). Obviously, the regioselectivity of AnaPT was disrupted by the unnatural alkyl donor **1**. A similar phenomenon was also observed in the presence of the larger alkyl donor **2**. Two products were identified in the enzyme assay of **I** with **2**. This is consistent with the observed higher flexibility of FgaPT2 and 5-DMATS toward **1** than **2** regarding the attack possibility.[4] In comparison to the assay with **1**, the C3-alkylated derivative **I-2d** is the main product in the assay with **2**, and the yield of C2-alkylated **I-2a** was approximately 42% of that of **I-2d**.

Scheme 1. Enzyme Reactions with the Natural Alkyl Donor

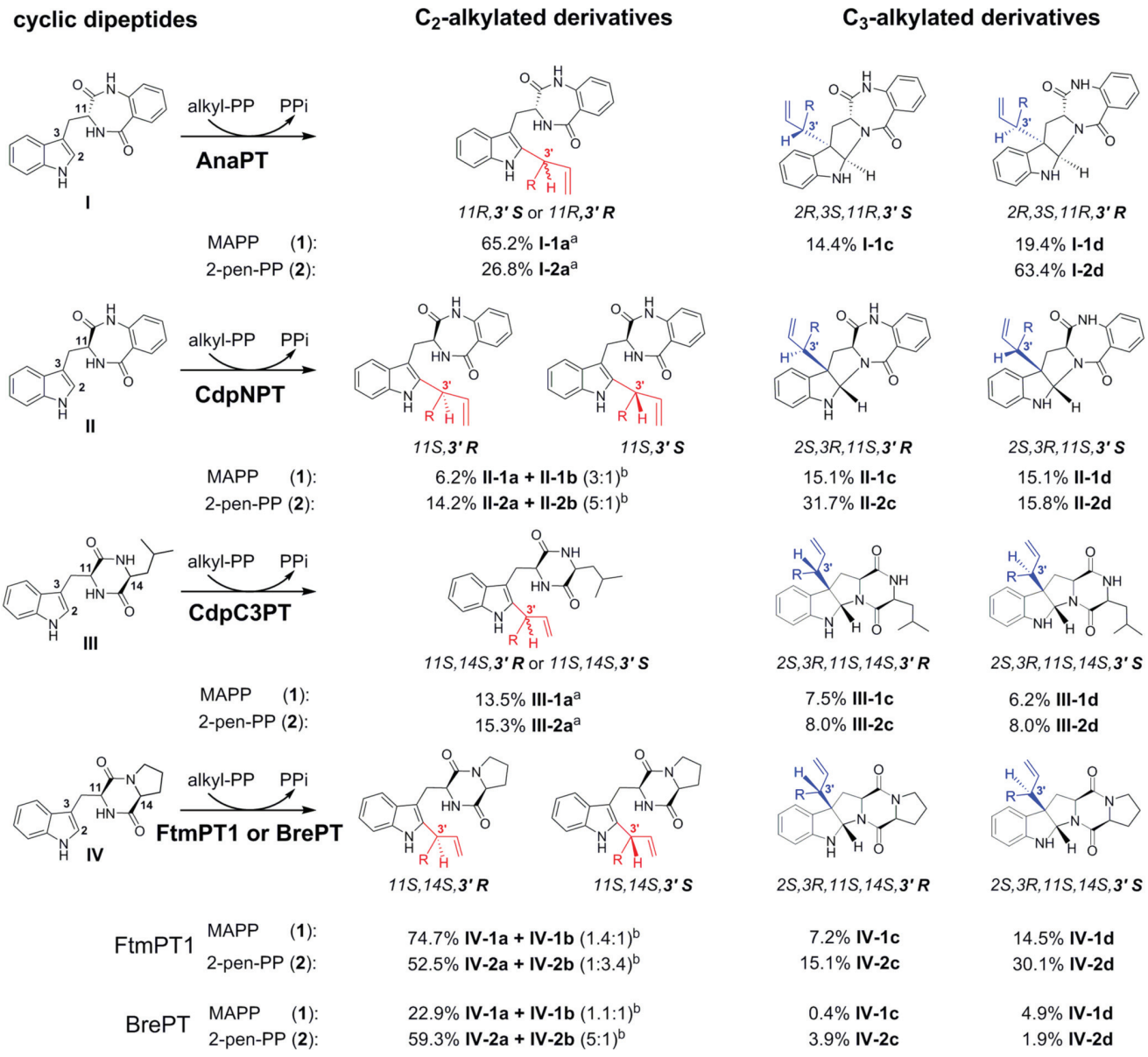


From enzyme assays of CdpNPT with **II** in the presence of **1** and **2**, two product peaks each were obtained after isolation on HPLC. Structure elucidation of the isolated peaks revealed that they contained two C2- and two C3-alkylated derivatives, respectively. Unfortunately, the two C2-alkylated derivatives **II-1a** and **II-1b**, or **II-2a** and **II-2b**, cannot be separated from each other, and their structures were elucidated as a mixture. The ratios of the product pairs given in Scheme 2 were calculated from integrals in their ^{1}H NMR spectra.

By comparing their ^{1}H NMR spectra, **II-1a** was unequivocally elucidated as the enantiomer of **I-1a**, and **II-1b** was proven to be a diastereomer of **II-1a**. The ^{1}H NMR spectra of **II-1c** and **II-1d** overlapped almost completely with those of **I-1c** and **I-1d**, respectively, proving that **II-1c** and **II-1d** are enantiomers of **I-1c** and **I-1d**, respectively, and

(5) Yin, W.-B.; Xie, X.-L.; Matuschek, M.; Li, S.-M. *Org. Biomol. Chem.* **2010**, *8*, 1133–1141.

Scheme 2. C2- and C3-Alkylated Derivatives from Enzyme Assays of AnaPT, CdpNPT, CdpC3PT, FtmPT1, and BrePT with **1** and **2** (R = CH_3, MAPP; CH_2CH_3, 2-pen-PP)



[a] Configuration at C3′ not determinable. [b] Assignment of stereoisomer **a** or **b** to the structures not possible.

therefore carry an alkyl moiety at the β-position. The structures of **II-2a**–**II-2d** were elucidated by comparison of the NMR spectra with those of **I-2a** and **I-2d**, respectively. In the assay of CdpNPT with **II** and **1**, the product yield of C2-alkylated derivatives is about 20% of that of C3-alkylated ones (Scheme 2). Using **2** instead of **1** as alkyl donor led to higher conversion yields, especially for C2-alkalyted derivatives, which reached 30% of that of C3-alkylated derivatives.

The third C3-prenyltransferase, CdpC3PT, like CdpNPT, also catalyzed β-prenylation of tryptophan-containing cyclic dipeptides in the presence of its natural alkyl donor, DMAPP (Scheme 1).[2d] Using **1** and **2** as alkyl donors, CdpC3PT was assayed in the presence of its best reported aromatic substrate, *cyclo*-L-Trp-L-Leu (**III**). One C2-reversely alkylated derivative each, **III-1a** or **III-2a**, and two C3-reversely β-alkylated derivatives, **III-1c**/**III-1d** or **III-2c**/**III-2d**, were identified in the reaction mixtures of **1** and **2**, respectively. In both assays, the product yield for the C2-alkylated derivative is nearly identical to that of the C3-alkylated derivatives together.

Taken together, AnaPT, CdpNPT, and CdpC3PT usually catalyze, in the presence of DMAPP, regioselective reverse prenylations at C3 of tryptophan-containing cyclic dipeptides.[2b–d] However, in the presence of the unnatural alkyl donors **1** and **2**, they catalyze both C2- and C3-reverse alkylations. The ratio of C2- to C3-alkylated derivatives was strongly dependent on the enzyme and substrates used

and varied from 1:5 in the reaction mixture of CdpNPT with **II** and **1** to 2:1 in that of AnaPT with **I** and **1**.

Identification of C2- and C3-alkylated products by three C3-prenyltransferases with **1** and **2** raised a challenge to investigate the behavior of C2-prenyltransferases. For this purpose, we used FtmPT1 and BrePT, which catalyze transfer reactions of a regular and a reverse prenyl moiety onto C2 of *cyclo*-L-Trp-L-Pro (**IV**; Scheme 1), respectively. HPLC analysis revealed the presence of the same product peaks in the reaction mixtures of FtmPT1 and BrePT with **1**, although the peak intensities differed. This phenomenon was also observed for the two enzyme assays with **2**. Product yields of 96.4% and 97.7% were calculated from enzyme assays with 20 μg of FtmPT1 for 16 h with **1** and **2**. From BrePT assays (50 μg, 16 h), the values were determined to be 69.7% for **1** and 32.2% for **2**.

Interpretation and comparison of ^{1}H NMR spectra with those of C2- and C3-prenylated derivatives[2d,e,6] proved indeed the presence of the same products in the corresponding enzyme assays of FtmPT1 and BrePT, i.e., two reversely C2- and two reversely C3-alkylated derivatives in each assay. This means that both C2-prenyltransferases catalyzed also C3-alkylation in the presence of the unnatural alkyl donors **1** and **2**. Interestingly, only reversely C2-alkylated derivatives were identified, even in the assays of the regular C2-prenyltransferase FtmPT1. It seems that the unnatural alkyl donors are less bulky and therefore more flexible than DMAPP. Thus, they can be easily reached by C2 and C3 of the indole ring. The reverse alkylations are likely the favored reactions. The total yield of C3-alkylated derivatives **IV-1c** and **IV-1d** in the assay of FtmPT1 with **1** reached nearly 30% of that of the C2-alkylated **IV-1a** and **IV-1b**. In the assay of FtmPT1 with **2**, the product yield of **IV-2c** and **IV-2d** was even 86% of that of **IV-2a** and **IV-2b**. The ratios of C3-alkylated to C2-alkylated derivatives were found to be 1:4 for BrePT with **1**, and 1:10 for BrePT with **2**.

Parallel alkylation at both the C2 and C3 positions of the indole ring by five prenyltransferases in the presence of unnatural alkyl donors breaks the regioselectivity of these enzymes. It seems that **1** and **2** were placed in the active sites of the tested enzymes in such positions, which allowed alkylations at both positions C2 and C3. Even in the presence of DMAPP, both C2 and C3 of **IV** are within a possible distance for prenylation. However, a regularly C2-prenylated intermediate would be more stable.[6] Breaking FtmPT1 regioselectivity was also observed for DMAPP with poorly accepted cyclic dipeptides.[7]

To learn more about the behavior of the five prenyltransferases in the presence of **1** and **2**, kinetic parameters were determined and calculated from Hanes–Woolf and Eadie–Hofstee transformations (Table 1). The observed reactions apparently followed Michaelis–Menten kinetics. In comparison to DMAPP, both **1** and **2** were comparably or even better accepted by the tested enzymes. However, the turnover numbers for **1** and **2** were much lower than those of DMAPP. For a given enzyme, no significant difference was observed between turnover numbers of **1** and **2**.

Table 1. Kinetic Parameters of the Tested Enzymes toward Their Alkyl Donors[a]

	DMAPP		MAPP (**1**)		2-pen-PP (**2**)	
	K_M	k_{cat}	K_M	k_{cat}	K_M	k_{cat}
AnaPT	156	1.5	52	0.020	80	0.011
CdpNPT	–	–	446	0.004	339	0.009
CdpC3PT	1400	0.098	1432	0.002	997	0.004
FtmPT1	74	5.57	49	0.007	63	0.015
BrePT	98	0.276	68	0.001	72	0.002

[a] K_M and k_{cat} values are in μM and s^{-1}, respectively. Data of DMAPP are from refs 2a,2b,2d,2e.

In conclusion, cyclic dipeptide prenyltransferases of the DMATS superfamily also accept unnatural DMAPP analogues and catalyze Friedel–Crafts alkylation. In comparison to the partial or total shift of alkylation position observed for the tryptophan prenyltransferases FgaPT2 and 5-DMATS with **1** and **2** in a previous study,[4] the regioselectivity was lost completely in the reactions of the cyclic dipeptide prenyltransferases. Regardless of the prenyltransferase, both C2- and C3-alkylated products were detected. The ratios of the two C2- to the two C3-stereoisomers and those of the C2- to C3-alkylated products strongly depend on the enzyme and alkyl donor used. Moreover, all main products exhibit substitution patterns of a reverse alkylation. Finally, the alkyl moieties at C3 have the same orientation as in the products with DMAPP.

The results presented in this study provide evidence for the fact that the members of the DMATS superfamily share not only structural similarity[2c,6] but also the ability to catalyze the same reactions, at least for the cyclic dipeptide prenyltransferases tested in this study. This feature can be considered as an important prerequisite for convenient creation of desired enzyme derivatives by site-directed mutagenesis.

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Supporting Information Available. Experimental procedures, determination of kinetic parameters, HR-MS, and detailed NMR data as well as NMR spectra. This material is available free of charge via Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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Breaking cyclic dipeptide prenyltransferase regioselectivity by unnatural alkyl donors

Supporting Information

Mike Liebhold[†], Xiulan Xie[§] and Shu-Ming Li*[,†]

[†]Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17a, 35037 Marburg, Germany , [§]Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany.

Experimental Procedures

Chemicals

Syntheses of dimethylallyl diphosphate (DMAPP), monomethylallyl diphosphate (MAPP; **1**) [*trans:cis*= 5:1] and 2-*trans*-pentenyl diphosphate (2-pen-PP; **2**) were carried out as described previously.[1,2] (*R*)- and (*S*)-benzodiazepindione (*cyclo*-L-Trp-D-anthranilic acid (**I**) and *cyclo*-L-Trp-L-anthranilic acid (**II**)) were synthesized according to the method described by Barrow and Sun.[3] *Cyclo*-L-Trp-L-Leu (**III**) was purchased from Bachem (Bubendorf, Switzerland). *Cyclo*-L-Trp-L-Pro (**IV**) was synthesized according to the method described by Caballero et al.[4]

Overproduction and purification of recombinant Proteins

The recombinant proteins FtmPT1, AnaPT, CdpNPT and CdpC3PT were prepared by cultivation of *Escherichia coli* M15 [pREP4] (Quiagen) cells harbouring the expression plasmid pAG12[5], pWY22[6], pHL5[7] and pWY25[8] respectively. The recombinant protein BrePT was prepared by cultivation of *Escherichia coli* SoluBL21™ (AMS Biotechnology) cells harbouring the expression plasmid pSY1.[9]

The cells were cultivated in 2.5 L Erlenmeyer flasks containing 1 L liquid teriffic broth (TB) medium and supplemented with kanamycin (50 µg ml^{-1}; BrePT) or with carbenicillin (50 µg ml^{-1}; other enzymes). The cultures were grown for 2-3 h at 37 °C and 220 rpm till an OD_{600} of 0.6 to 0.7 was reached. After addition of IPTG at final concentrations of 0.1 (FtmPT1, BrePT), 0.5 (AnaPT, CdpC3PT) or 1.0 mM (CdpNPT) IPTG, the cells were further incubated at 30 °C (FtmPT1, BrePT) or 37 °C (other enzymes) for 5 h (BrePT), 6 h (AnaPT, CdpC3PT) or 16 h (FtmPT1, CdpNPT). Protein preparation and purification were carried out as described previously.[5,6,8-10]

Assays for determination of enzyme activities

The reaction mixtures (100 µl) for determination of the enzyme activities contained 1 mM aromatic subtrate, 10 mM $CaCl_2$, 2 mM **1** or **2**, 1.0-1.5% (v/v) glycerol, 2.5% (v/v) dimethyl sulfoxide (DMSO), 50 mM Tris pH 7.5 and 20 µg (AnaPT and FtmPT1) or 50 µg (CdpC3PT, CdpNPT and BrePT) of purified recombinant protein. The reaction mixtures were incubated at 37 °C for 16 h. For HPLC analysis on RP18 column, the reactions were then terminated with 100 µl MeOH. Protein was removed by centrifugation at 17.000 x g for 15 min. For HPLC analysis on silica gel column the reactions were terminated and extracted twice with two volumes of ethyl acetate, dried in a vacuum centrifuge and dissolved in 200 µl $CHCl_3$.

Enzyme assays for isolation and structure elucidation

Assays for isolation of the enzyme products were carried out in large scales (10 ml-40 ml) containing aromatic substrate (1 mM), alkyl diphosphates (2 mM), $CaCl_2$ (10 mM), glycerol (1.0%-1.5% v/v), DMSO (2.5% v/v), Tris HCl (50 mM pH 7.5) and with a minimum of 0.1 mg and up to 0.5 mg protein per ml assay. After incubation for 16 h at 37 °C, the reaction mixtures were terminated and extracted with two volumes of ethyl acetate. The assays were then further extracted two more times with another two volumes and one volume of ethyl acetate. The combined ethyl acetate phases were concentrated using a rotary evaporator. ^1H

NMR spectra were taken for the residues to determine product yields. After evaporation of the NMR solvent, the samples were dissolved in a mixture of DMSO/MeOH and subjected to preparative HPLC equipped with RP 18 columns.

Enzyme assays for determination of kinetic parameters

Assays for determination of kinetic parameters contained 1 mM of aromatic substrate, 10 mM $CaCl_2$, 1.0-1.5% (v/v)glycerol, 2.5% (v/v) DMSO, 50 mM Tris HCl pH 7.5 and alkyl diphosphates in final concentrations of at least 0.002 mM and up to 20 mM. Protein amount and incubation time varied between 6 µg to 30 µg and 30 min to 180 min at an incubation temperature of 37 °C. The reactions were then terminated with 100 µl MeOH. Protein was removed by centrifugation at 17.000 x g for 15 min.

HPLC analysis and isolation of enzyme products for structure elucidation

The enzyme products were routinely analyzed by HPLC on an Agilent series 1200 by using a MultoSpher 120 RP-18 column (250 x 4 mm, 5µ C+S Chromatographie Service, Langerwehe, Germany) or MultoHigh100 Si column (250 x 4 mm, 5µ C+S Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 ml min^{-1}. For reverse phase chromatography, water (solvent A) and methanol (solvent B) were used as solvents for analysis and isolation of the enzyme products. A linear gradient of 40-100% (v/v) solvent B in A in 15 min was used for analysis of the enzyme products of FtmPT1, BrePT and CdpC3PT. The column was then washed with 100% solvent B in A for 5 min and equilibrated with 40% solvent B in A for another 5 min. For AnaPT and CdpNPT, a linear gradient of 55-100% (v/v) solvent B in A in 15min was used. The column was then washed with 100% solvent B in A for 5 min and equilibrated with 55% solvent B in A for another 5 min. Detection was carried out on a photo diode array detector.

For normal phase chromatography, chloroform (solvent C) and methanol (solvent B) were used as solvents for analysis and isolation of the enzyme products. A linear gradient of 0-6% (v/v) solvent B in C in 30 min was used for analysis of the enzyme products of AnaPT, CdpNPT, FtmPT1 and BrePT. For CdpC3PT a linear gradient of 0-8% (v/v) solvent B in C was used. The column was then washed with 50% solvent B in C for 5 min and equilibrated with 0% solvent B in C for another 5 min. Detection was carried out by photo diode array detector

The products of BrePT and AnaPT with **1** as well as AnaPT and C3PT with **2** were isolated by repeated chromatography on a MultoSpher 120 RP18 column (250 x 10 mm, 5 µm). Gradients of 50-90% (v/v) in 20 min, 75-85% (v/v) in 20 min and 60-100% (v/v) solvent B in A in 20 min at 2.5 ml min^{-1} were used for BrePT, AnaPT with **1** as well as AnaPT and C3PT with **2** respectively. For the isolation of the other enzyme products, different gradients in consecutive steps were used. The products of FtmPT1 with **1** and **2** were isolated using a gradient of 50-90% (v/v) solvent B in A in 20 min followed by further isolation with a gradient of 60-100% (v/v) solvent B in A in 30 min on RP18 column. The isolation of products of CdpNPT with **1** was carried out with gradients of 60-100% (v/v) in 20 min and 75-90% (v/v) in 30 min, those with **2** with gradients of 60-100% (v/v) in 15 min and 75-85% (v/v) in 30 min. The products of BrePT with **2** were isolated first with a gradient of 50-90% (v/v) in 20 min and then with 60-90% (v/v) solvent B in A in 20 min. CdpC3PT products with **1** were isolated by using a gradient of 50-90% (v/v) in 20 min and 75-100% (v/v) solvent B in A in 30 min. After each run, the columns were washed with 100% solvent B in A for 5 min followed by 5min of equilibration with the corresponding condition at the beginning of each run.

If necessary, further separation by normal phase chromatography was carried out with MultoHigh100 Si column (250 x 4 mm, 5µm C+S Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 ml min^{-1}. Gradients of 0-4% up to 0-10% (v/v) solvent B in C were used. After each run, the columns were washed with 50% solvent B in C for 5 min followed by 5min of equilibration with the corresponding condition at the beginning of each run.

NMR and MS analyses

^{1}H NMR spectra were recorded on a JEOL ECA-500 or a Bruker Avance 500 MHz spectrometer. Two-dimensional spectra HSQC and HMBC were recorded on a Bruker Avance 500 MHz spectrometer. Chemical shifts were referred to the signal of $CHCl_3$-*d* at 7.26 ppm for ^{1}H NMR spectra as well as 77.0 ppm for ^{13}C NMR spectra. Spectra were processed with MestRENova 5.2.2. The isolated compounds were also analyzed by electron impact mass spectrometry (EI-MS) or electrospray ionization mass spectrometry (ESI-MS) on an Auto SPEC (Micromass Co. UK Ltd.).

NMR assignments

Reverse alkyl moiety was proven by the chemical shifts and coupling patterns of the signals of H-1' and H-2', *e. g.* H-1´ at δ_H 5.16 (1H, dt, 10.3,1.3) and 5.13 (1H, dt, 17.3, 1.3) as well as H-2´ at δ_H 6.06 (1H, ddd, 17.3, 10.3, 5.7) of **I-1a**. The protons of H-1' displayed constants for a *trans* and a *cis* coupled proton of H-2´, which was also reflected by the signals of H-2'. This proved the presence of a terminal olefinic group.

For determination of the configuration at position C-2 and C-3 of C3 alkylated derivatives, the coupling constants of H-11 were taken in consideration. For the α-configuration of products with **I** and β-configuration with **II**, a double doublet with couplings of around 9 and 7.5 Hz (or a triplet with approximate 8 Hz) were observed as for the products of DMAPP with **I** and **II**.[11] For the β-configuration in products from tryptophan-containing diketopiperazines, a double doublet with coupling constants of about 11 and 6 Hz were detected.[12]

C2- and C3-alkylated compounds were easily distinguished by comparison of the chemical shifts of the signal for H-3' of the alkyl moieties. With **1** as substrate, chemical shifts of 3.93 to 3.83 ppm and 2.56 to 2.47 ppm were observed for C2- and C3-alkylated products, respectively. In the case of **2**, chemical shifts were detected at 3.61 to 3.52 ppm for C2- and 2.18 to 2.03 ppm for C3-alkylated products. In a similar manner, the C3' stereoisomers themselves can be also distinguished. For this purpose, the relative chemical shifts of H-1', H-2' and H-4' for products with **1** or H-1', H-2' and H-5' for products with **2** had to be compared. In the case of D-Trp as indole moiety in **II**, the signals of H-1' and H-2' of *3'S*-isomer were relatively up-field shifted, in comparison to those of the *3'R*-isomer, while the signals for H-4' and H-5'were downfield shifted. This phenomenon was also observed for products of **1** and **2** with **II** and other tryptophan-containing cyclic dipeptides labeled as *3'S* and *3'R*.

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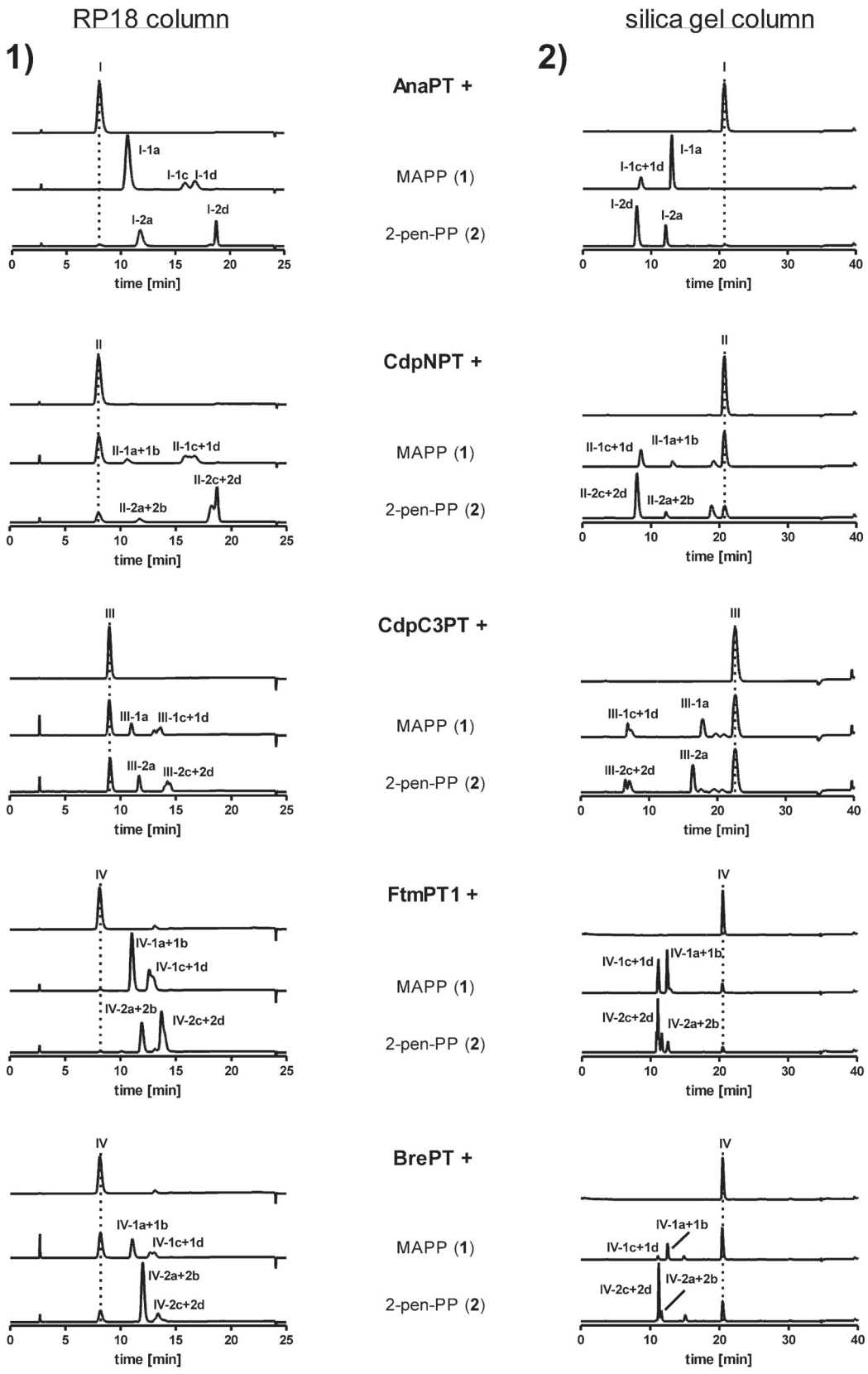


Figure S1. HPLC analysis on RP18 column **1)** and silica gel column **2)** of reaction mixtures of AnaPT, CdpNPT, CdpC3PT, FtmPT1 and BrePT with **1** or **2** at 37 °C and 16 h.

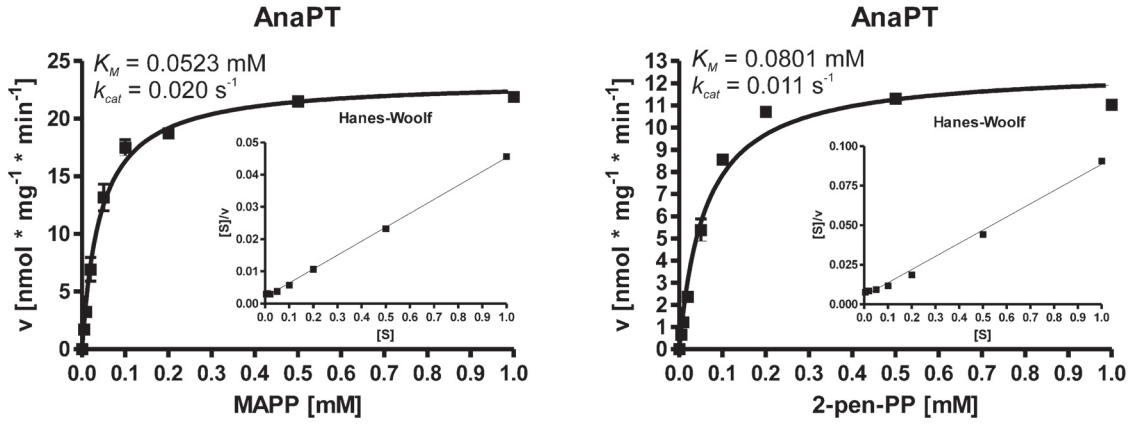


Figure S2. Dependence of the product formation of the AnaPT reaction on the presence of **1** (MAPP) and **2** (2-pen-PP).

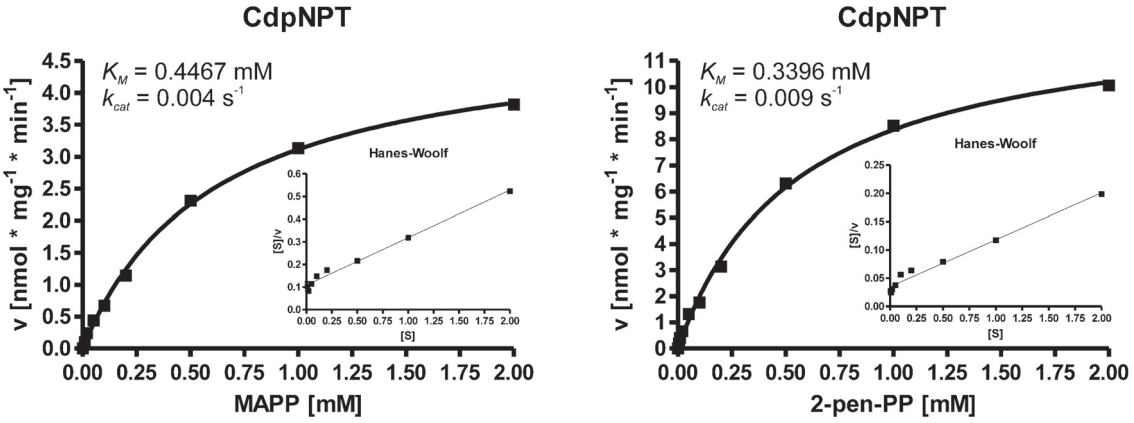


Figure S3. Dependence of the product formation of the CdpNPT reaction on the presence of **1** (MAPP) and **2** (2-pen-PP).

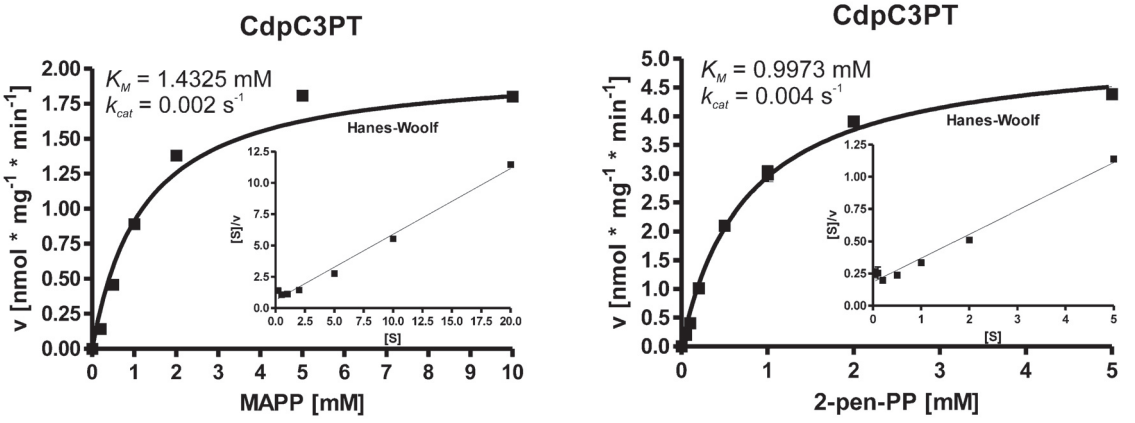


Figure S4. Dependence of the product formation of the CdpC3PT reaction on the presence of **1** (MAPP) and **2** (2-pen-PP).

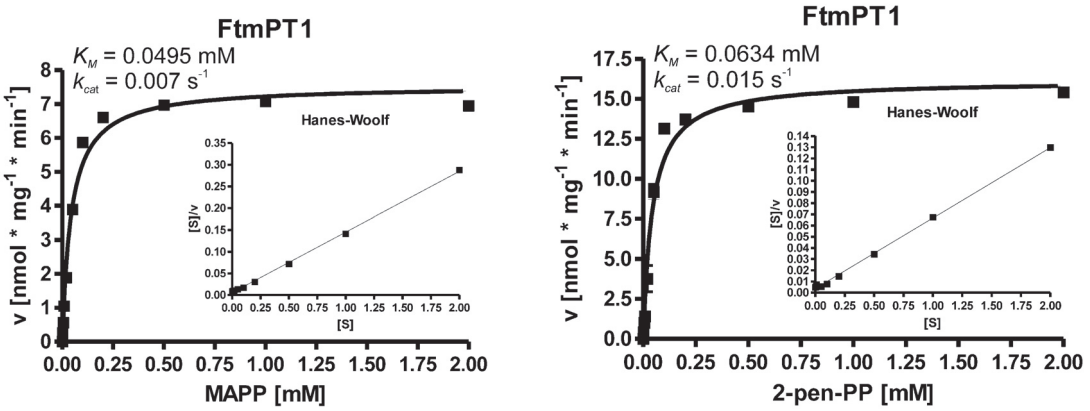


Figure S5. Dependence of the product formation of the FtmPT1 reaction on the presence of **1** (MAPP) and **2** (2-pen-PP).

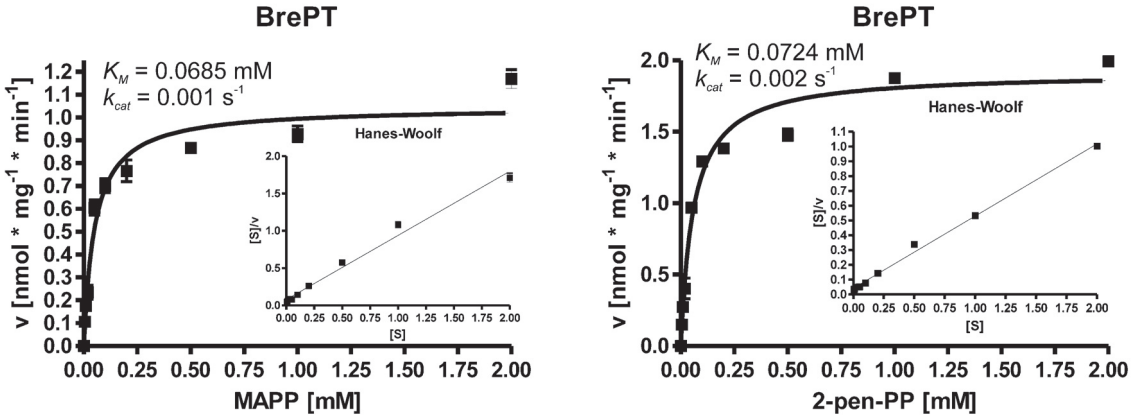


Figure S6. Dependence of the product formation of the BrePT reaction on the presence of **1** (MAPP) and **2** (2-pen-PP).

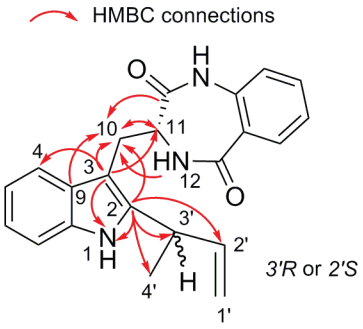


Figure S7. HMBC correlations of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**).

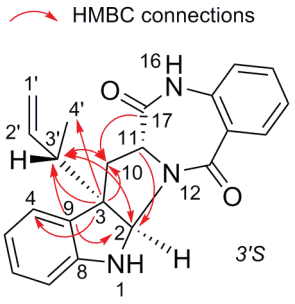


Figure S8. HMBC correlations of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**).

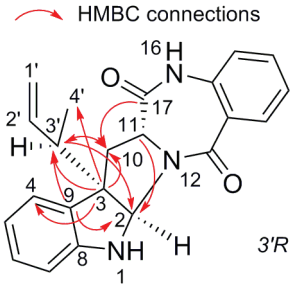


Figure S9. HMBC correlations of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**).

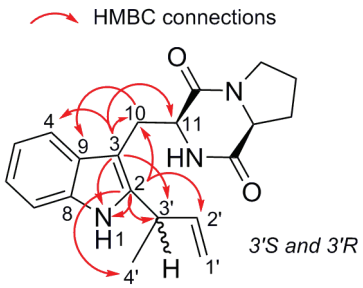


Figure S10. HMBC correlations of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**).

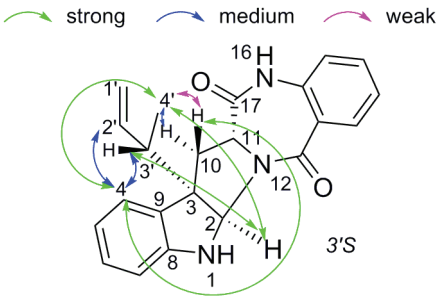


Figure S11. NOESY correlations of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**).

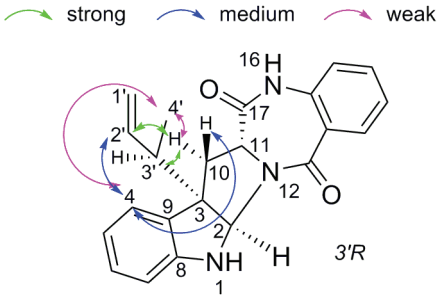


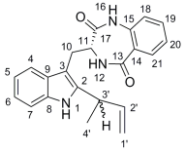
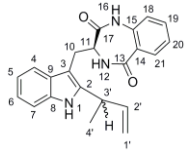
Figure S12. NOESY correlations of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**).

Table S13. MS Data of the enzymatic products of AnaPT, CdpNPT, CdpC3PT, FtmPT1 and BrePT with **1** and **2**.

	MAPP					2-Pen-PP				
				ESI- MS data					ESI- MS data	
	compound	*alkylation position*	*chemical formula*	*calculated*	*measured*	*compound*	*alkylation position*	*chemical formula*	*calculated*	*measured*
AnaPT	I-1a	C-2	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	382.15 $[M+Na]^+$	I-2a	C-2	$C_{23}H_{23}N_3O_2$	373.18 $[M]^+$	396.09 $[M+Na]^+$
	I-1c	C-3	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	382.15 $[M+Na]^+$	-	-	-	-	-
	I-1d	C-3	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	382.15 $[M+Na]^+$	I-2d	C-3	$C_{23}H_{23}N_3O_2$	373.18 $[M]^+$	374.25 $[M+H]^+$
CdpNPT	II-1a	C-2	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	360.14 $[M+H]^+$ [a]	II-2a	C-2	$C_{23}H_{23}N_3O_2$	373.18 $[M]^+$	374.19 $[M+H]^+$ [e]
	II-1b	C-2	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	360.14 $[M+H]^+$ [a]	II-2b	C-2	$C_{23}H_{23}N_3O_2$	373.18 $[M]^+$	374.19 $[M+H]^+$ [e]
	II-1c	C-3	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	360.14 $[M+H]^+$	II-2c	C-3	$C_{23}H_{23}N_3O_2$	373.18 $[M]^+$	374.14 $[M+H]^+$
	II-1d	C-3	$C_{22}H_{21}N_3O_2$	359.16 $[M]^+$	360.13 $[M+H]^+$	II-2d	C-3	$C_{23}H_{23}N_3O_2$	373.18 $[M]^+$	374.11 $[M+H]^+$
CdpC3PT	III-1a	C-2	$C_{21}H_{27}N_3O_2$	353.21 $[M]^+$	354.20 $[M+H]^+$	III-2a	C-2	$C_{22}H_{29}N_3O_2$	367.23 $[M]^+$	368.27 $[M+H]^+$
	III-1c	C-3	$C_{21}H_{27}N_3O_2$	353.21 $[M]^+$	354.14 $[M+H]^+$	III-2c	C-3	$C_{22}H_{29}N_3O_2$	367.23 $[M]^+$	368.15 $[M+H]^+$
	III-1d	C-3	$C_{21}H_{27}N_3O_2$	353.21 $[M]^+$	354.19 $[M+H]^+$	III-2d	C-3	$C_{22}H_{29}N_3O_2$	367.23 $[M]^+$	368.18 $[M+H]^+$
FtmPT1	IV-1a	C-2	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	360.17 $[M+Na]^+$ [b]	IV-2a	C-2	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.15 $[M+H]^+$ [f]
	IV-1b	C-2	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	360.17 $[M+Na]^+$ [b]	IV-2b	C-2	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.15 $[M+H]^+$ [f]
	IV-1c	C-3	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	338.21 $[M+H]^+$	IV-2c	C-3	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.13 $[M+H]^+$
	IV-1d	C-3	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	338.15 $[M+H]^+$	IV-2d	C-3	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.14 $[M+H]^+$
BrePT	IV-1a	C-2	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	338.16 $[M+H]^+$ [c]	IV-2a	C-2	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.20 $[M+H]^+$ [g]
	IV-1b	C-2	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	338.16 $[M+H]^+$ [c]	IV-2b	C-2	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.20 $[M+H]^+$ [g]
	IV-1c	C-3	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	338.09 $[M+H]^+$ [d]	IV-2c	C-3	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.24 $[M+H]^+$ [h]
	IV-1d	C-3	$C_{20}H_{23}N_3O_2$	337.18 $[M]^+$	338.09 $[M+H]^+$ [d]	IV-2d	C-3	$C_{21}H_{25}N_3O_2$	351.19 $[M]^+$	352.24 $[M+H]^+$ [h]

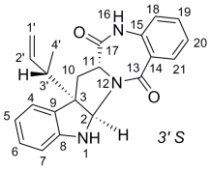
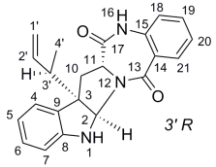
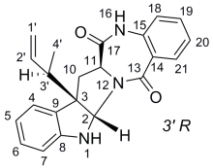
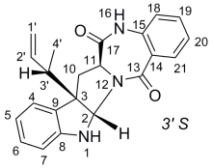
[a-h] MS data with same letter were obtained from mixtures.

Table S14. ^{1}H NMR Data of C2-alkylated products of AnaPT and CdpNPT with MAPP

Comp.	AnaPT **I-1a** 		CdpNPT **II-1a** 	**II-1b**
Pos.	δ_H, *multi*, *J*	δ_C	δ_H, *multi*, *J*	δ_H, *multi*, *J*
1	7.94, br. s	-	7.65, br. s	7.65, br. s
2	-	139.0	-	-
3		104.8		
4	7.40, d, 7.7	117.7	7.38, dd, 8.0, 0.8	7.42, dd, 7.7, 0.7
5	7.04, ddd, 7.9, 7.1, 0.9	119.6	7.04, ddd, 8.0, 7.1, 1.0	7.06, ddd, 8.0, 7.1, 1.0 *
6	7.13, ddd, 8.1, 7.1, 1,0	121.7	7.14, ddd, 8.2, 7.1, 1.2	7.14 *
7	7.31, d, 7.9	110.7	7.32, dt, 8.1, 0.8	7.31, dt, 8.0, 0.9 *
8		135.4		
9		127.9		
10	3.47, dd, 15.1, 5.7	23.3	3.47, dd, 15.2, 5.6	3.44, dd, 15.2, 6.0 *
	3.21, dd, 15.1, 9.2		3.21, dd, 15.2, 9.4	3.24, dd, 15.2, 9.0 *
11	4.20, dt 9.2, 5.7	51.9	4.19, ddd, 9.4, 5.6, 4.9	4.17, ddd, 0.1, 5.9, 5.1 *
12	6.30, d, 5.0	-	5.98, d, 4.9 *	5.95, d, 5.2 *
13		168.2		
14		125.3		
15		135.3		
16	8.29, s	-	7.92, br. s	7.92, br. s
17		171.6		
18	6.99, dd, 7.9, 0.6	120.7	6.95, dd, 8.0, 1.0	6.94, dd, 8.1, 1.1
19	7.49, ddd, 8.0, 7.4, 1.6	132.9	7.49, ddd, 8.1, 7.4, 1.6	7.49 *
20	7.24, td 7.6, 1.0 §	125.1	7.24, ddd, 7.8 7.5, 1.1 §	7.24 *
21	7.89, dd 7.9, 1.5	131.5	7.88, dd, 7.9, 1.7	7.90, dd, 8.0, 1.7 *
1‘	5.16, dt, 10.3, 1.3	115.1	5.21, dt, 10.3, 1.3	5.08, ddd, 10.4, 1.8, 1.4
	5.13, dt, 17.3, 1.3		5.15, ddd, 17.2, 1.6, 1.2	5.05, ddd, 17.3, 1.9, 1.3
2‘	6.06, ddd, 17.3, 10.3, 5.7	140.8	6.09, dd, 17.2, 10.3, 5.7	6.00, ddd, 17.3, 10.4, 5.1 *
3’	3.93, m	34.0	3.93, m	3.93 *
4’	1.42, d, 7.2	19.0	1.44, d, 7.1	1.46, d, 7.2
$CDCl_3$	7.26, s	77.0	7.26, s	7.26, s

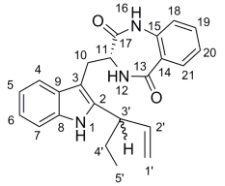
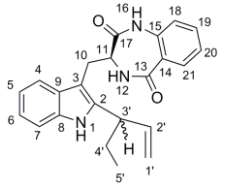
* signals overlapping with those of **II-1a**; § signals overlapping with those of solvent

Table S15. ^{1}H NMR Data of C3β-alkylated products of AnaPT and CdpNPT with MAPP

Comp.	AnaPT				CdpNPT	
	I-1c 		**I-1d** 		**II-1c** 	**II-1d** 
Pos.	δ_H, *multi*, *J*	δ_C	δ_H, *multi*, *J*	δ_C	δ_H, *multi*, *J*	δ_H, *multi*, *J*
1	-	-	-	-		
2	5.49, s	82.7	5.49, s	83.3	5.49, s	5.49, s
3		57.4		57.4		
4	7.13, d, 7.4	124.2	7.10, d, 7.3 [a]	124.4	7.14, dt, 7.4, 0.6	7.11, ddd 7.4, 1.2, 0.6 [c]
5	6.73, t, 7.4	118.5	6.72, td, 7.4, 0.9	118.5	6.73, td, 7.4, 1.0	6.72, td, 7.4, 1.0
6	7.08, t, 7.7, 1.0	128.5	7.08, td, 7.6, 1.1 [a]	128.5	7.08, td, 7.7, 1.2	7.08, td, 7.6, 1.3 [c]
7	6.63, d, 7.8	109.2	6.65, d, 7.8	109.3	6.64, dd, 7.8, 0.5	6.65, ddd, 7.8, 0.9, 0.6
8		148.8		148.8		
9		130.4		130.3		
10	3.29, dd, 13.8, 7.3	35.2	3,25, dd, 14.0, 7.5	34.7	3.29, dd, 13.8, 7.3	3.25, dd, 14.0, 7.5
	2.50, dd, 13.8, 8.7		2.47, dd, 14.0, 8.6		2.51, dd, 13.8, 8.8 [b]	2.47, dd, 14.0, 8.7
11	4.05, dd, 8.7, 7.3	57.0	4.02, dd,8.6, 7.5	56.9	4.05, dd, 8.8, 7.3	4.02, dd, 8.7, 7.5
12	-	-	-	-	-	-
13		166.6		166.6		
14		126.7		126.7		
15		133.9		133.9		
16	7.66, s	-	7.66, s		7.68, br. s	7.64, br. s
17		169.8		169.7		
18	6.90, d, 7.9	120.4	6.90, dd, 8.0, 0.5	120.4	6.91, dd, 8.0, 0.7	6.90, dd, 8.0, 1.0
19	7.45, td, 7.9, 1.5	132.6	7.44, td, 7.7, 1.6	132.5	7.45, dtd, 8.0, 7.4, 1.6	7.45, ddd, 8.0, 7.4, 1.6
20	7.23, td, 7.6, 0.8	125.2	7.22, td, 7.7, 1.0	125.2	7.23, ddd, 7.9, 7.4, 1.1	7.23, ddd, 7.9, 7.4, 1.1
21	7.86, dd, 7.9, 1.3	131.2	7.85, dd, 7.9, 1.4	131.2	7.86, dd, 7.9, 1.6	7.84, dd, 7.9, 1.6
1‘	5.07, d, 17.1	115.7	5.20, d, 17.1	116.4	5.07, ddd, 17.1, 1.7, 0.8	5.20, ddd, 17.1, 1.7, 1.1
	5.02, dd, 10.2, 1.5		5.16, dd, 10.3, 1.2		5.01, dd, 10.3, 1.7	5.16, ddd, 10.3, 1.8, 0.7
2‘	5.61, ddd, 17.1, 10.2, 8.7	139.6	5.99, ddd, 17.1, 10.3, 8,4	139.2	5.60, ddd, 17.1, 10.3, 8.7	5.99, ddd, 17.1, 10.3, 8.4
3’	2.56, m	45.2	2.53, m	44.4	2.56, m [b]	2.53, m
4’	1.18, d 6.9	16.2	1.02, d, 6.8	16.2	1.18, d, 6.9	1.01, d, 6.8
$CDCl_3$	7.26, s	77.0	7.26, s	77.0	7.26, s	7.26, s

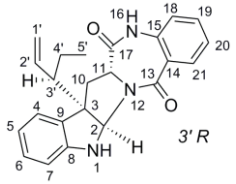
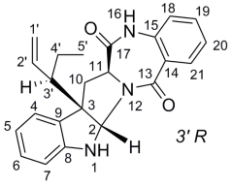
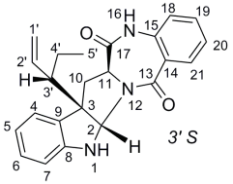
[a,b,c] signals with same letters overlapping each other

Table S16. ^{1}H NMR Data of C2-alkylated products of AnaPT and CdpNPT with 2-pen-PP

Comp.	AnaPT **I-2a** 	CdpNPT **II-2a** 	**I-2d**
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
1	7.63, br. s	7.63, br. s	7.61, br.s
2	-	-	-
4	7.38, d, 8.0	7.38, dd, 7.9, 1.0	7.44, d, 8.0
5	7.04, ddd, 8.0, 7,1, 1.0	7.04, ddd, 8.0, 7.1, 1.0	7.08, ddd, 8.0, 7.1, 1.0
6	7.15, ddd, 8.2, 7.1, 1.1	7.15, ddd, 8.2, 7.1, 1.2	7.15 *
7	7.32, dt, 8.1, 0.8	7.32, dt, 8.1, 0.9	7.30, m
10	3.46, dd, 15.4, 5.1	3.45, dd, 15.3, 5.1	3.43, dd, 15.2, 6.2
	3.21, dd, 15.4, 10.1	3.21, dd, 15.3, 10.1	3.25, dd, 15.2, 8.9
11	4.19 dt, 10.1, 5.1	4.19, dd, 10.1, 5.1	4.15, m *
12	6.00, d, 5.2	5.97, d, 4.6	5.99, d, 4.8
16	7.91 br. s	7.91, br. s	7.87, br. s
18	6.96, dd, 8.0, 0.9	6.96, dd, 8.1, 1.0	6.94, m
19	7.49, ddd, 8.1, 7.4, 1.6	7.49, ddd, 8.1, 7.4, 1.6	7.49 *
20	7.24, td, 7.5, 1.1 §	7.24, ddd, 7.9, 7.5, 1.1	7.24 *
21	7.85, dd, 8.0, 1.4	7.85, dd, 7.9, 1.6	7.89, dd, 7.9, 1.7
1‘	5.21, dt, 10.3, 1.3	5.21, dt, 10.3, 1.3	5.02, dt, 10.4, 1.4
	5.12, dt, 17.3, 1.3	5.12, dd, 17.2, 1.3	4.95, dt, 17.3, 1.4
2‘	6.04, ddd, 17.3, 10.3, 6.5	6.04, ddd, 17.2, 10.3, 6.5	5.93, 17.3, 10.4, 6.0
3‘	3.60, m	3.61, m	3.61 *
4‘	1.86 m	1.87, m	1.87 *
	1.75 m	1.75, m	1.75 *
5‘	0.89, t, 7.3	0.89, t, 7.4	0.93, t, 7.3
$CDCl_3$	7.26, s	7.26, s	7.26, s

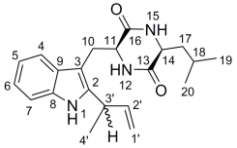
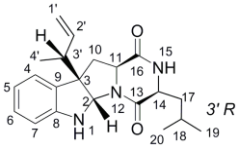
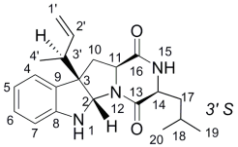
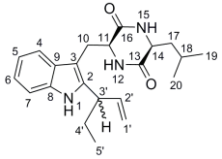
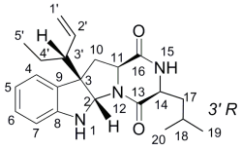
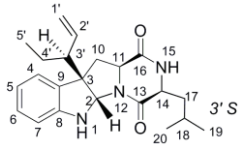
* signals overlapping with those of **II-2a**; § signals overlapping with those of solvent

Table S17. ^{1}H NMR Data of C3β-alkylated products of AnaPT and CdpNPT with 2-pen-PP

Comp.	AnaPT **I-2d** 	CdpNPT **II-2c** 	**II-2d** 
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
1	-	-	-
2	5.50, s	5.49, s	5.50, s
4	7.12, dd, 7.4, 0.7	7.13, dd, 7.4, 0.6	7.12, dd, 7.4, 0.7
5	6.73, td, 7.4, 1.0	6.72, td, 7.4, 0.9	6.73, td, 7.4, 1.0
6	7.08, td, 7.8, 1.2	7.08, td, 7.8, 1.2	7.08, td, 7.7, 1.2
7	6.66, d, 7.8	6.63, d, 7.8	6.65, d, 7.8
10	3.22, dd, 14.0, 7.5 2.44, dd, 14.0, 8.7	3.29, dd, 13.8, 7.2 2.53, dd, 13.8, 8.8	3.22, dd, 14.0, 7.6 2.44, dd, 14.0, 8.6
11	4.00, dd, 8.4, 7.7	4.05, dd, 8.8, 7.2	3.99, dd, 8.6, 7.6
12	-	-	-
16	7.67, br. s	7.88, br.s	7.74, br s
18	6.90, dd, 8.1, 0.7	6.92, d, 8.0	6.90, dd, 8.1, 0.9
19	7.44, ddd, 8.0, 7.4, 1.6	7.45, ddd, 7.9, 7.4, 1.6	7.44, ddd, 8.0, 7.4, 1.6
20	7.22, ddd, 8.1, 7.4, 1.1	7.23, td, 7.9, 1.0	7.22, ddd, 7.9, 7.4, 1.1
21	7.84, dd, 7.9, 1.6	7.86, dd, 8.0, 1.4	7.83, dd, 7.9, 1.6
1‘	5.25, m 5.25, m	5.11, dd, 10.1, 2.0 5.07, dd, 16.8, 2.0	5.25, m 5.25, m
2‘	5.78, dt,17.7, 9.7	5.29, dt, 16.8, 10.1	5.78, dt, 17.9, 9.5
3‘	2.18, m	2.15, m	2.18, m
4‘	1.79, dqd, 13.5, 7.4, 2.8 1.02, dqd, 13.5, 7.4, 11.5	1.72, dqd, 13.2, 7.3, 2.4 1.42, dqd, 13.2, 7.3, 11.5	1.80, dqd, 13.6, 7.4, 2.8 1.02, dqd, 13.6, 7.4, 11.5
5‘	0.78, t, 7.3	0.86, t, 7.3	0.78, t, 7.4
$CDCl_3$	7.26, s	7.26, s	7.26, s

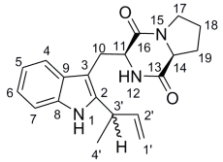
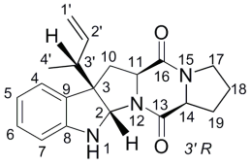
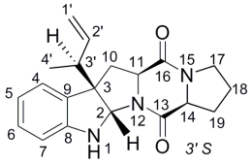
[§] signals overlapping with those of solvent

Table S18. ^{1}H NMR Data of C2- and C3β-alkylated products CdpC3PT with MAPP and 2-pen-PP

Comp.	CdpC3PT (MAPP) III-1a	III-1c	III-1d	CdpC3PT (2-pen-PP) III-2a	III-2c	III-2d
						
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
1	7.93, br. s	-	-	7.92, br.s	-	-
2	-	5.40, s	5.37, s	-	5.40, br. s	5.38, br. s
4	7.55, ddt, 7.8, 1.4, 0.7	7.09 *	7.09, d, 7.6 [b]	7.54, ddt, 7.8, 1.2, 0.6	7.12, d, 7.5	7.12 **
5	7.11, ddd, 7.9, 7.1, 1.1	6.78, td, 7.7, 0.8 *	6.76, td 7.5, 0.8	7.12, ddd, 7.9, 7.1, 1.1	6.77, td, 7.5, 1.0	6.76, td, 7.5, 1.0 **
6	7.17, ddd, 7.9, 7.1, 1,2	7.12 *	7.12, d, 7.3 [b]	7.18, ddd, 8.1, 7.1, 1.2	7.09, td 7.6, 1.9	7.10, td , 7.7, 1.9 **
7	7.31, ddd, 8.0, 1.0, 0.9	6.58, dd, 7.8, 0.9 *	6.59, dd, 8.1, 0.8	7.33, dt, 8.0, 0.9	6.58, d, 7.8	6.60, d 7.8 **
10	3.51, dd, 14.8, 3.7	2.66, dd, 12.7, 6.2 *	2.65, dd, 12.9, 6.2	3.58, dd, 14.7, 3.5	2.69, dd 12.7, 6.3	2.63, dd, 13.0, 6.1
	3.15, dd, 14.8, 9.3	2.35, dd, 12.7, 11.2	2.29, dd, 12.9, 11.2	3.06, dd, 14.7, 10.1	2.36, dd, 12.7, 11.3	2.25, dd, 13.0, 11.2
11	4.31, dddd, 9.3, 3.7, 2.1, 1.2	4.03, m *	4.01, m	4.30, dddd, 10.1, 3.5, 2.0, 1.2	4.03, ddd, 11.3, 6.3, 1.7	3.99, m **
12	5.75, br. s [a]	-	-	5.72, s	-	-
14	3.91, dddd, 10.0, 3.8, 2.3, 1.2	3.96 *	3.96, m	3.93, dddd, 9.8, 3.4, 2.0, 1.2	3.96, m	3.96 **
15	5.90, br. s [a]	5.65 *	5.65, br. s	5.94, s	5.66, br. s	5.66 **
17	1.64, ddd, 13.5, 10.0, 3.8	2.04 *	2.04, m §	1.73, m	2.03, m	2.03 **
	1.14, ddd, 13.5, 10.0, 4.6	1.54 *	1.54 §	1.34, ddd, 13.5, 9.8, 4.5	1.55, m [d]	1.55 **
18	1.55 §	1.68 *	1.68, m	1.66, m	1.69, m [e]	1.69 **
19	0.86, d, 6.5	1.00 *	1.00, d 6.6	0.93, d, 6.5	1.00, d, 6.6 [f]	1.00 **
20	0.85, d, 6.5	0.92 *	0.92, d, 6.6	0.90, d, 6.5	0.92, dd, 6.6, 1.0	0.92 **
1‘	5.20, ddd, 10.4, 1.6, 1.2	5.09, ddd, 17.1, 1.6, 0.8*	5.12, ddd, 10.4, 1.6, 0.6 [c]	5.20, dt, 10.4, 1.3	5.12, dd, 10.2, 2.1	5.21, dd, 10.1, 1.8
	5.16, ddd, 17.3, 1.7, 1.2	5.01, dd, 10.2, 1.7 *	5.10, ddd, 17.0, 1.5, 1.0 [c]	5.14, dt, 17.1, 1,3	5.09, dd, 16.8, 2.0	5.14, ddd, 17.0, 1.8, 0.5
2‘	6.06, ddd, 17.3, 10.4, 5.5	5.54, 17.1, 10.2, 8.9	5.88, ddd, 17.0, 10.4, 8.3	6.02, ddd, 17.1, 10.4, 6.5	5.28,dt, 16.8, 10.2	5.70, dt, 17.0, 10.1
3‘	3.81, m	2.49, m *	2.47, m	3.52, dtt, 8.2, 6.8, 1.4	2.12, m	2.11, m **
4‘	1.43, d, 7.1	1.07, d, 6.9	0.97, d, 6.8	1.86, m	1.51, m [d]	1.73, m [e]
	-	-	-	1.73, m	1.34, ddd, 13.3, 11.5, 7.2	0.97, m [f]
5‘	-	-	-	0.89, t, 7.0	0.80, t, 7.4	0.76, t, 7.4
$CDCl_3$	7.26, s	7.26, s	7.26, s	7.26, s	7.26, s	7.26, s

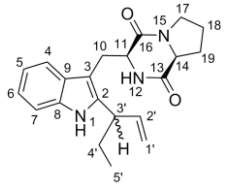
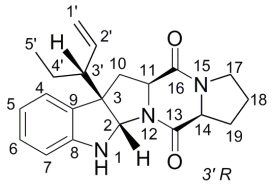
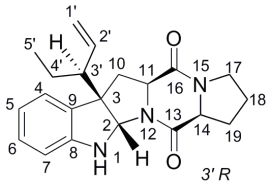
* signals overlapping with those of **III-1d**; ** signals overlapping with those of **III-2c;** § signals overlapping with those of solvent; [a-f] signals with same are overlapping each other

Table S19. ^{1}H NMR Data of C2- and C3β-alkylated products of FtmPT1 and BrePT with MAPP

Comp.	FtmPT1 and BrePT					
	IV-1a		**IV-1b**		**IV-1c**	**IV-1d**
						
Pos.	δ_H, multi, J	δ_C	δ_H, multi, J	δ_C	δ_H, multi, J	δ_H, multi, J
1	7.96 br. s	-	7.97, s *	-	-	-
2	-	138.9	-	138.9	5.37, s, br.	5.38, br. s (weak)
3		104.6		104.6		
4	7.50, d, 7.9	117.8	7.50 *	117.8	7.13, d, 6.8 [b]	7.12, d, 7.7
5	7.11, t, 7.6	119.9	7.11 *	119.9	6.80, t 7.4	6.80, t 7.2 (weak)
6	7.18, t, 7.5	122.1	7.18 *	122.1	7.11, t, 7.6 [b]	7.14 (weak)
7	7.33, d, 8.1	110.8	7.33 *	110.8	6.62, d, 6.7	6.65-6.68, br. s (weak)
8		135.3		135.3		
9		127.6		127.6		
10	3.72, dd, 15.1, 3.9	25.2	3.67, dd, 14.4, 3.8 [a]	25.3	2.66, dd, 12.9, 6.5	2.65, dd, 13.2, 6.4
	2.96, dd, 15.1, 11.4		3.01, dd, 14.4, 10,8		2.38, dd, 12.9, 11.1	2.33, dd, 13.2, 11.1 [d]
11	4.38, m	54.4	4.38 *	54.4	4.08, m	4.06, m [e]
12	5.66, s	-	5.63, s	-	-	-
13		169.1		169.1		
14	4.06, m	59.0	4.06 *	59.0	4.08, m	4.09, m [e]
16		165.6		165.6		
17	3.68 [a]	45.2	3.68 *	45.2	3.56, ddd, 11.8, 8.9, 7.3 [c]	3.56, ddd, 11.9, 8.8, 7.4
	3.59, ddd, 11.8, 9.2, 2.8		3.59 *		3.51, ddd, 11.8, 8.6, 3.3 [c]	3.51, ddd, 11.9, 8.7, 3.4
18	2.06, m	22.4	2.06 *	22.4	2.04, m	2.04, m
	1.91, m		1.91 *		1.90, m	1.89, m
19	2.33, m	28.1	2.33 *	28.1	2.32, dtd, 9.5, 6.8, 2.8	2.32, m [d]
	2.06, m		2.06 *		2.12, m	2.12, m
1‘	5.23, dd, 10.3, 0.9	115.4	5.12, dd, 10.3, 0.7	114.7	5.07, dd, 17.0, 0.8	5.11, ddd, 10.3, 1.6, 0.6 [f]
	5.18, dd, 17.2, 0.7		5.06, dd, 17.2, 0.6		5.00, dd, 10.2, 1.6	5.10, ddd, 17.0, 1.5, 1.1 [f]
2‘	6.10, ddd, 17.2, 10.3, 5.8	140.2	6.00, ddd, 17.2, 10.3, 5.4	140.3	5.54, ddd, 17.0, 10.2, 8.8	5.88, ddd, 17.0, 10.3, 8.3
3‘	3.83, m	33.9	3.83 *	33.9	2.49, dq, 8.8, 6.9	2.47, m
4‘	1.42, d, 7.1	19.4	1.48, d, 7.1	19.1	1.08, d, 6.9	0.97, d, 6.8
$CDCl_3$	7.26, s	77.0	7.26, s	77.0	7.26, s	7.26, s

* signals overlapping with those of **IV-1a**; [a-f] signals with same letters overlapping each other

Table S20. [1]H NMR Data of C2- and C3β-alkylated products of FtmPT1 and BrePT with 2-pen-PP

Comp.	FtmPT1 and BrePT			
	IV-2a / **IV-2b** 		**IV-2c**  3′*R*	**IV-2d**  3′*R*
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
1	8.03, br. s	8.06, br. s	-	-
2	-	-	5.35, s	5.35, s
4	7.51, dd, 7.8, 1.0	7.51 *	7.12, ddd, 7.4, 1.2, 0.6 [d]	7.13, dd, 7.4, 0.6 [e]
5	7.11, ddd, 8.1, 7.1, 1.1	7.12, ddd, 8.1, 7.1, 1.1	6.77, td, 7.5, 1.0	6.78, t, 7.4
6	7.19, ddd, 8.1, 7.1, 1.2	7.18, ddd, 8.1, 7.1, 1.2	7.10, dd, 7.6, 1.3 [d]	7.11, td, 7.7, 1.2 [e]
7	7.34, dt, 8.1, 0.9	7.33, dt, 8.0, 0.9	6.59, d, 7.8	6.61, d, 7.5
10	3.75, dd, 15.1, 3.6	3.66, dd, 15.2, 3.9 [a]	2.69, dd, 12.9, 6.5	2.62, dd, 13.2, 6.3
	2.94, dd, 15.1, 11.7	3.01, dd, 15.2, 11.4	2.38, dd, 12.9, 11.1	2.29, dd, 13.2, 11.1 [f]
11	4.38, ddd, 11.7, 3.6, 1.3	4.34, ddd, 11.4, 3.9, 1.2	4.03, m	4.03, ddd, 11.1, 6.3, 1.5 [g]
12	5.65, br. s	5.66, br. s	-	-
14	4.06, m	4.07, m *	4.07, m	4.07, m [g]
17	3.68, m [a]	3.68*	3.55, ddd, 11.9, 8.9, 7.3	3.55, ddd, 11.8, 8.8, 7.5
	3.61, m	3.61 *	3.51, ddd, 11.9, 8.6, 3.3	3.50, ddd, 118. 8.5, 3.4
18	2.07, m	2.07 *	2.03, m §	2.03, m §
	1.92, m	1.92 *	1.89, m	1.89, m
19	2.33, m	2.33 *	2.32, dtd, 12.8, 6.9, 2.8	2.31, m [f]
	2.07, m	2.07 *	2.11, m §	2.12, m §
1‘	5.22, dt, 10.3, 1.2	5.07, dt, 10.3, 1.4	5.10, dd, 10.2, 2.0	5.20, dd, 10.1, 1.9
	5.14, dt, 17.2, 1,2	4.97, dt, 17.1, 1.4	5.08, ddd, 16.8, 2.0, 0.4	5.14, ddd, 17.0, 1.9, 0.6
2‘	6.04, ddd, 17.2, 10.3, 6.8	5.94, ddd, 17.1, 10.3, 6.3	5.27, ddd, 16.8, 10.2, 9.8	5.69, ddd, 17.0, 10.1
3‘	3.52, m	3.52 *	2.15-2.00 §	2.15-2.00 §
4‘	1.85, m [b,c]	1.92, m [b]	1.55, dqd, 13.3, 7.4, 2.6	1.73, dqd, 13.6, 7.5, 2.9
	1.72, m [c]	1.79, m [c]	1.33, dqd, 13.3, 7.3, 11.5	0.97, dqd, 13.6, 7.4, 11.5
5‘	0.88, t, 7.4	0.96, t, 7.4	0.79, t, 7.4	0.75, t, 7.4
$CDCl_3$	7.26, s	7.26, s	7.26, s	7.26, s

* signals overlapping with those of **IV-2a**; § signals overlapping with those of solvent; [a-g] signals with same letters are overlapping each other

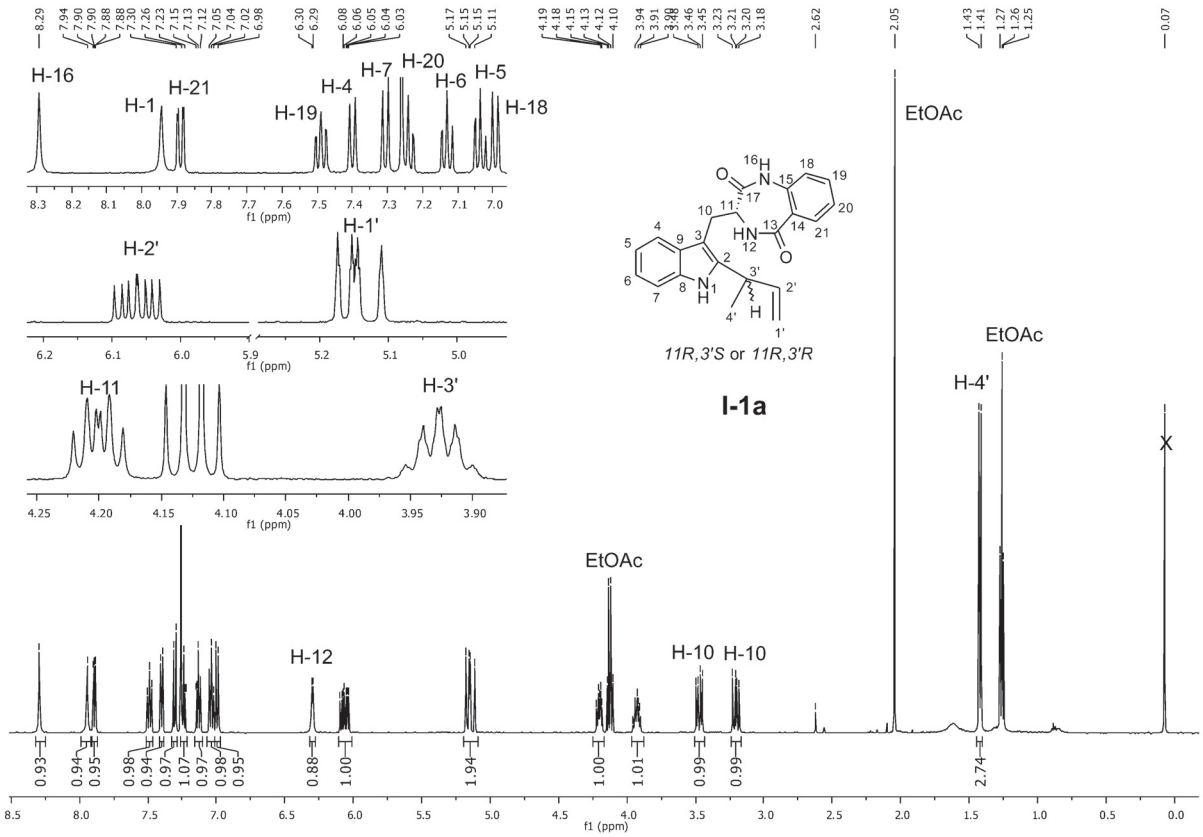


Figure S21. ^{1}H NMR spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

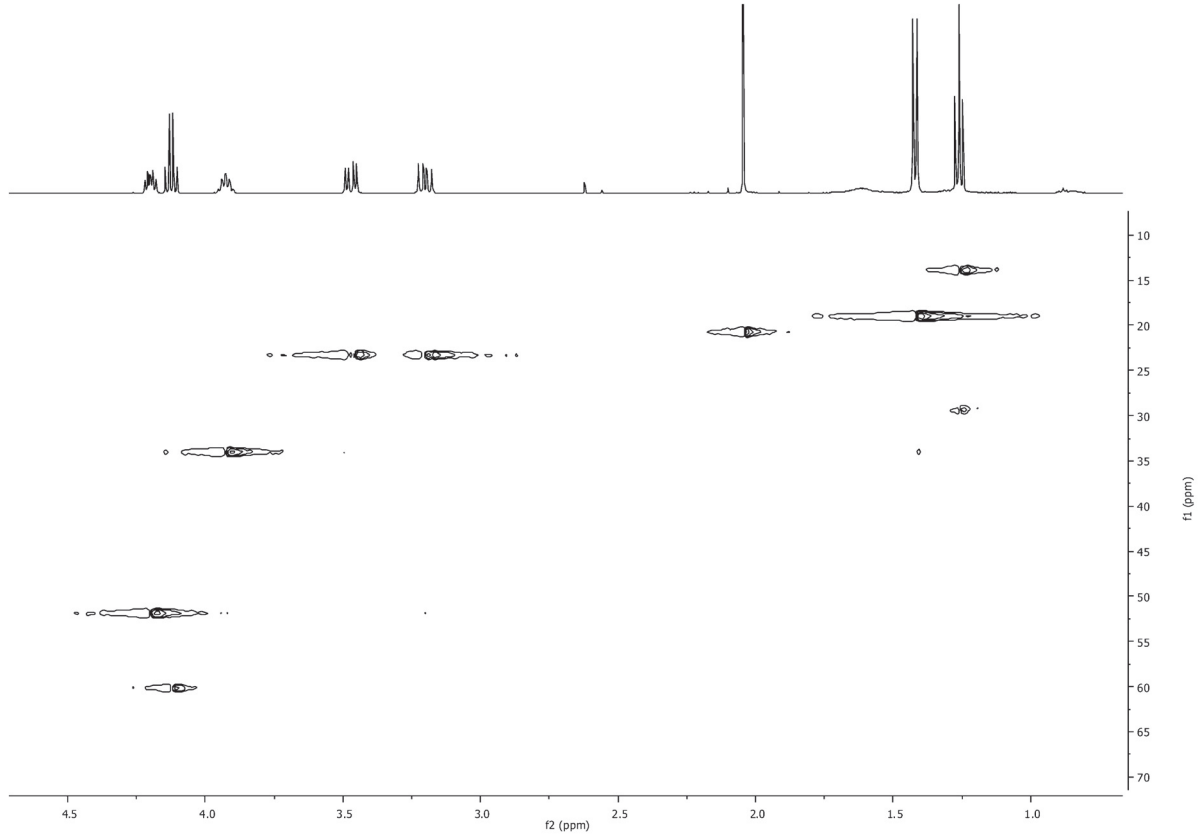


Figure S22. HSQC spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

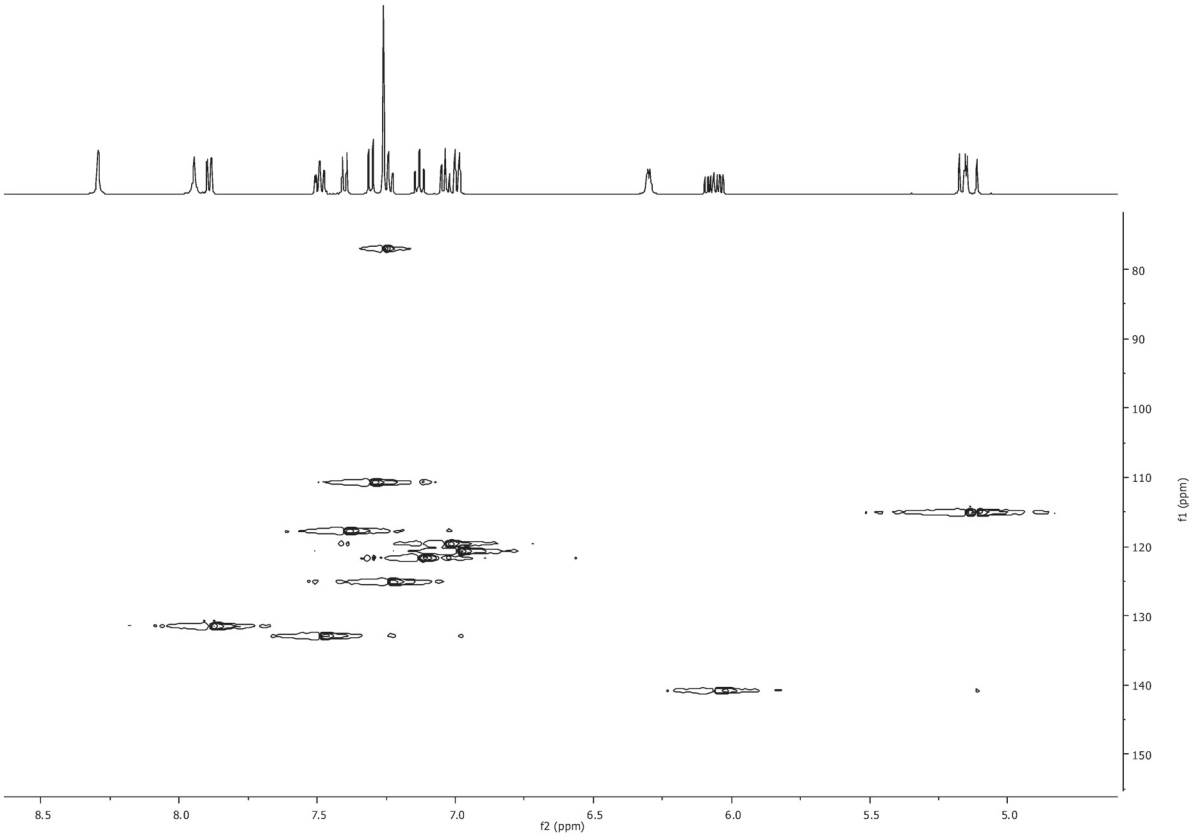


Figure S23. HSQC spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

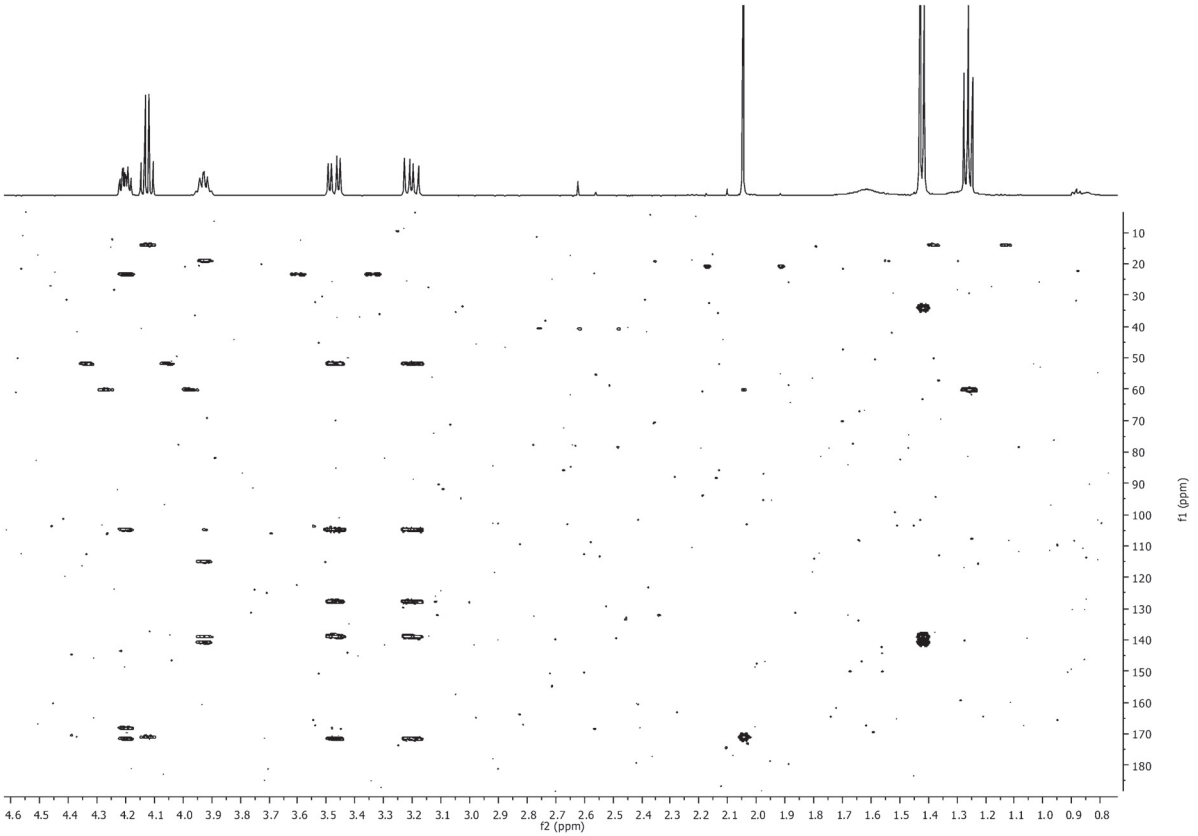


Figure S24. HMBC spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

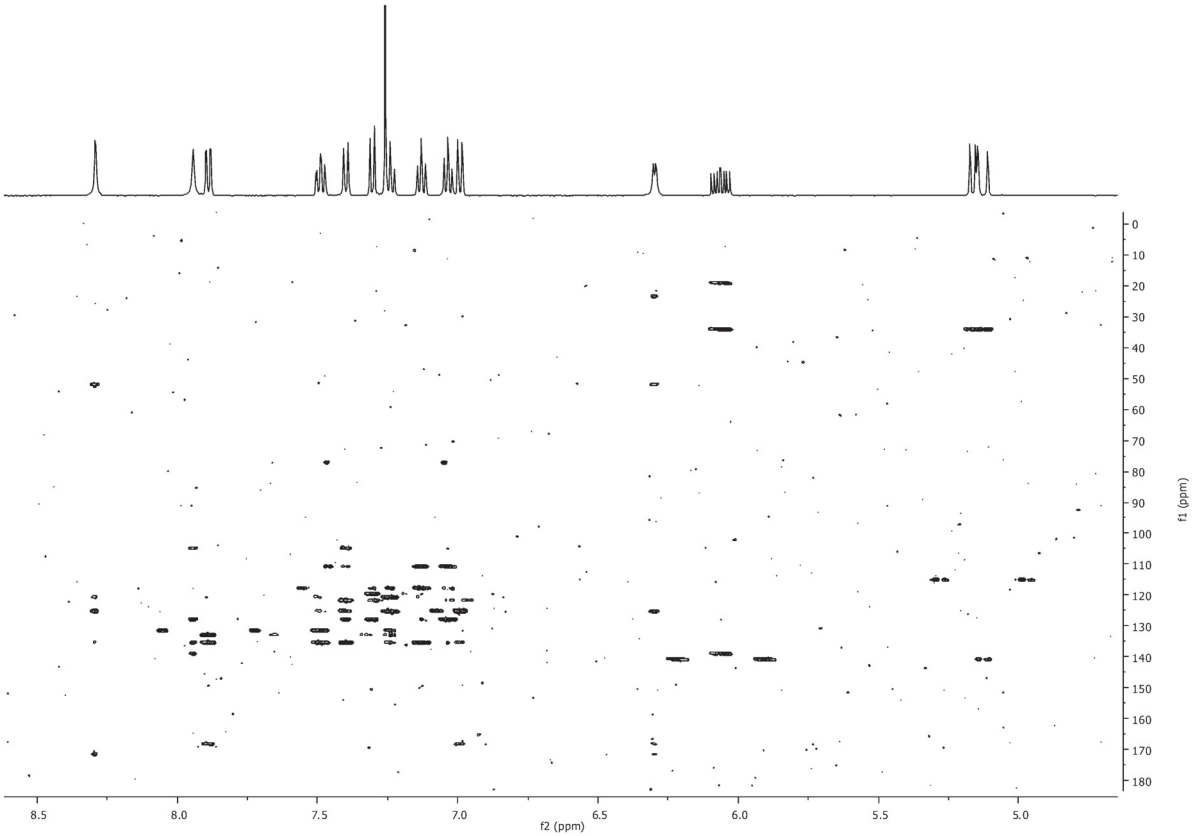


Figure S25. HMBC spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

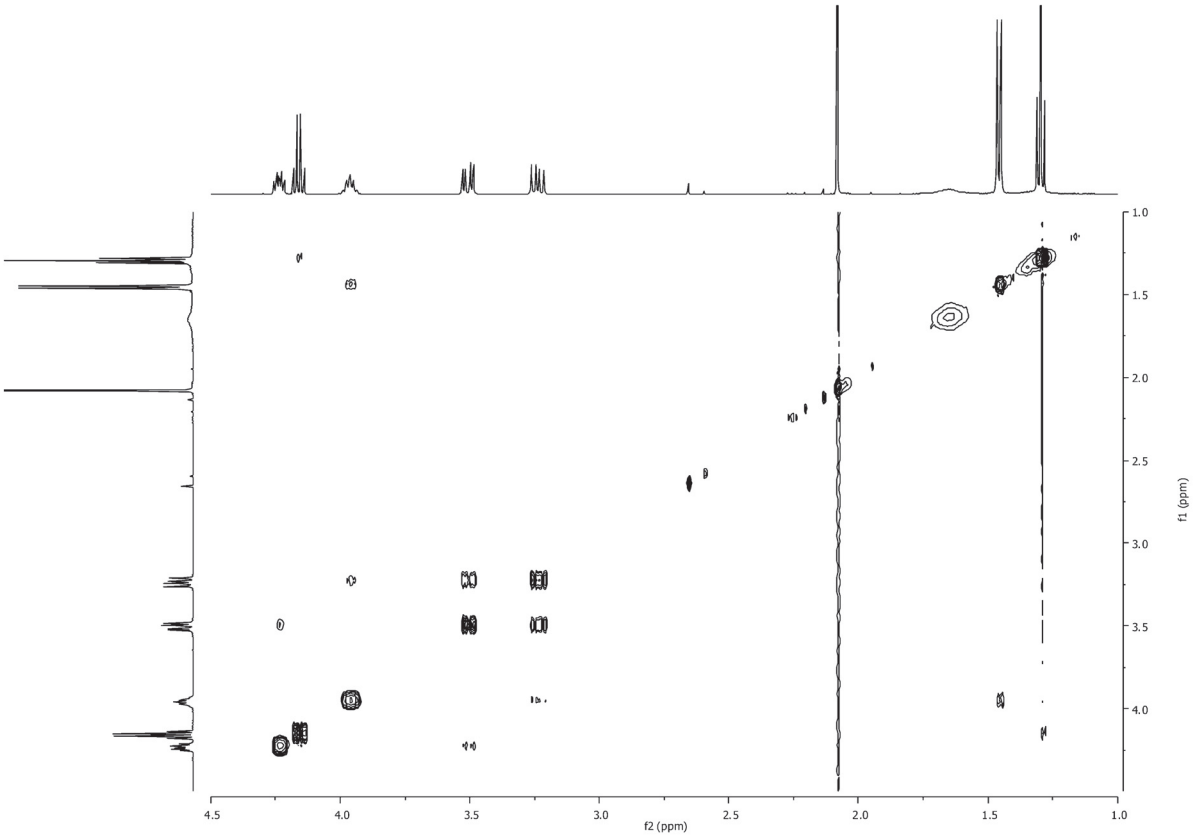


Figure S26. NOESY spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

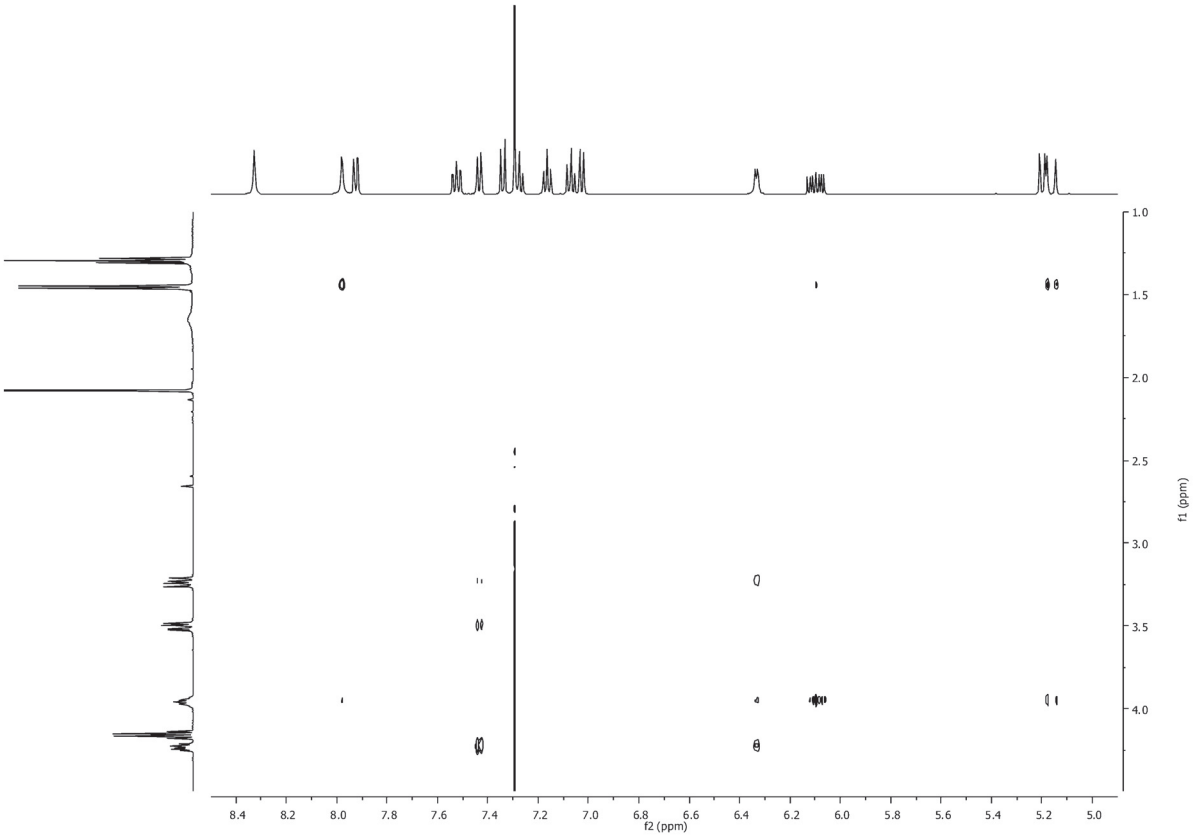


Figure S27. NOESY spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

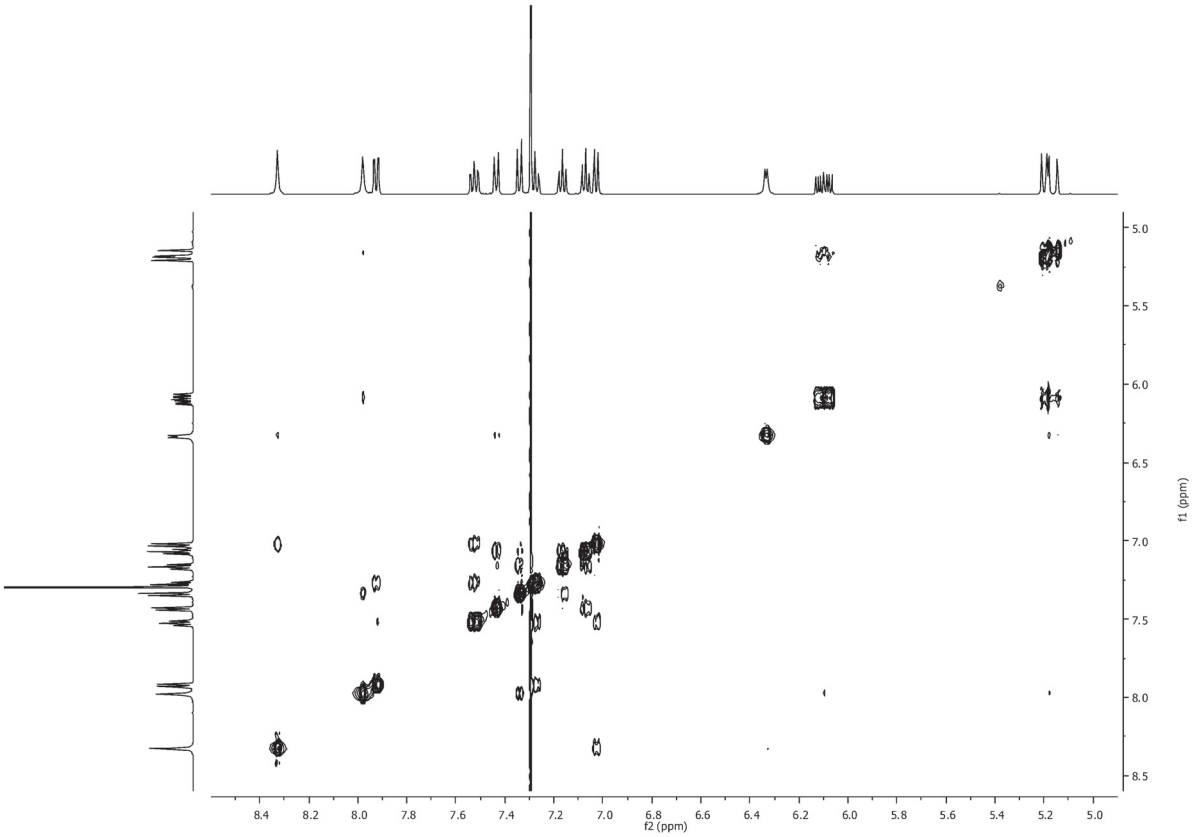


Figure S28. NOESY spectrum of C2-(3'-butenyl-)-(*R*)-benzodiazepindione (**I-1a**) in $CDCl_3$.

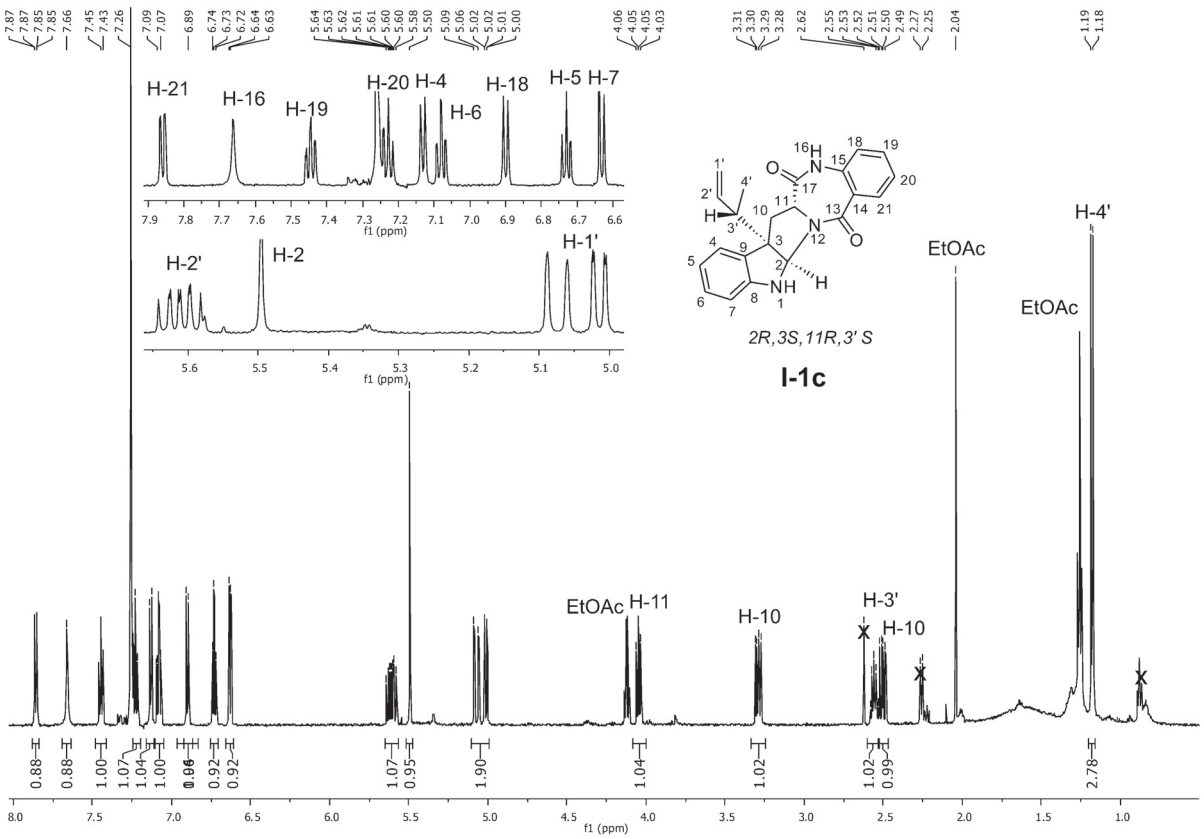


Figure S29. ^{1}H NMR spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

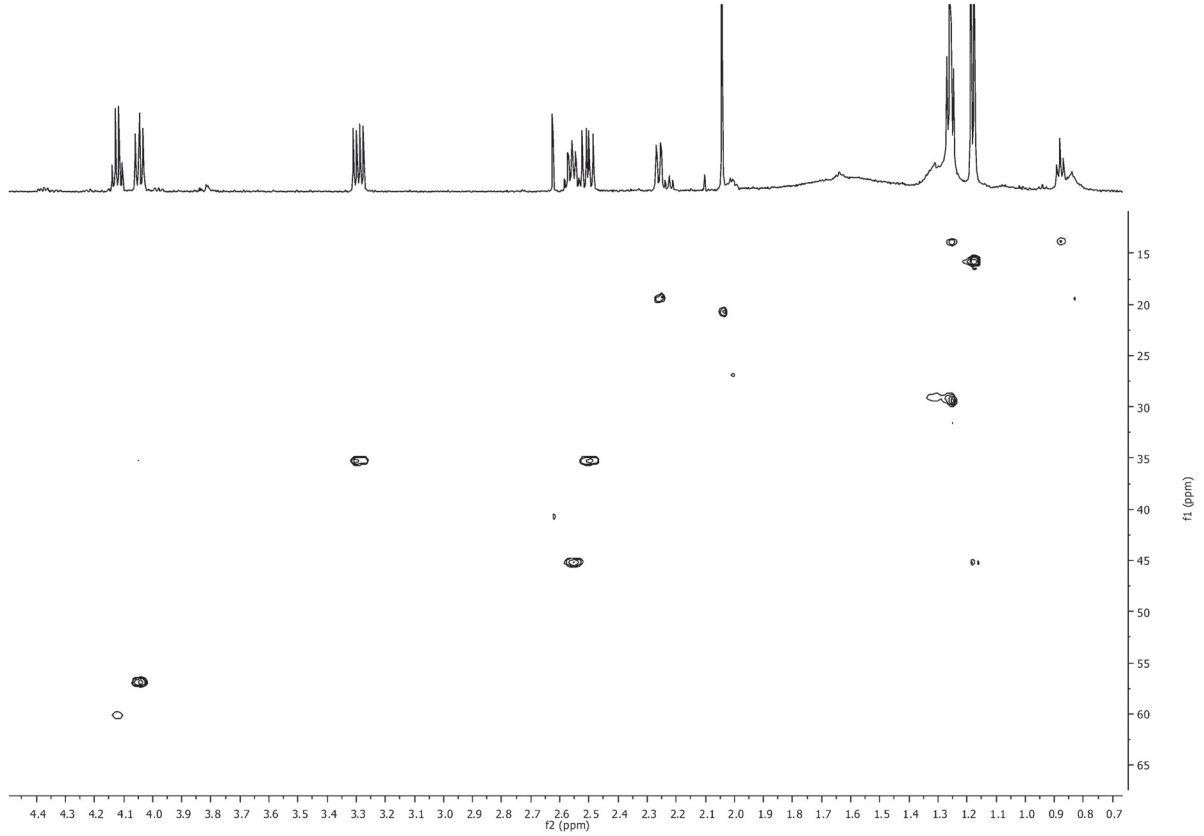


Figure S30. HSQC spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

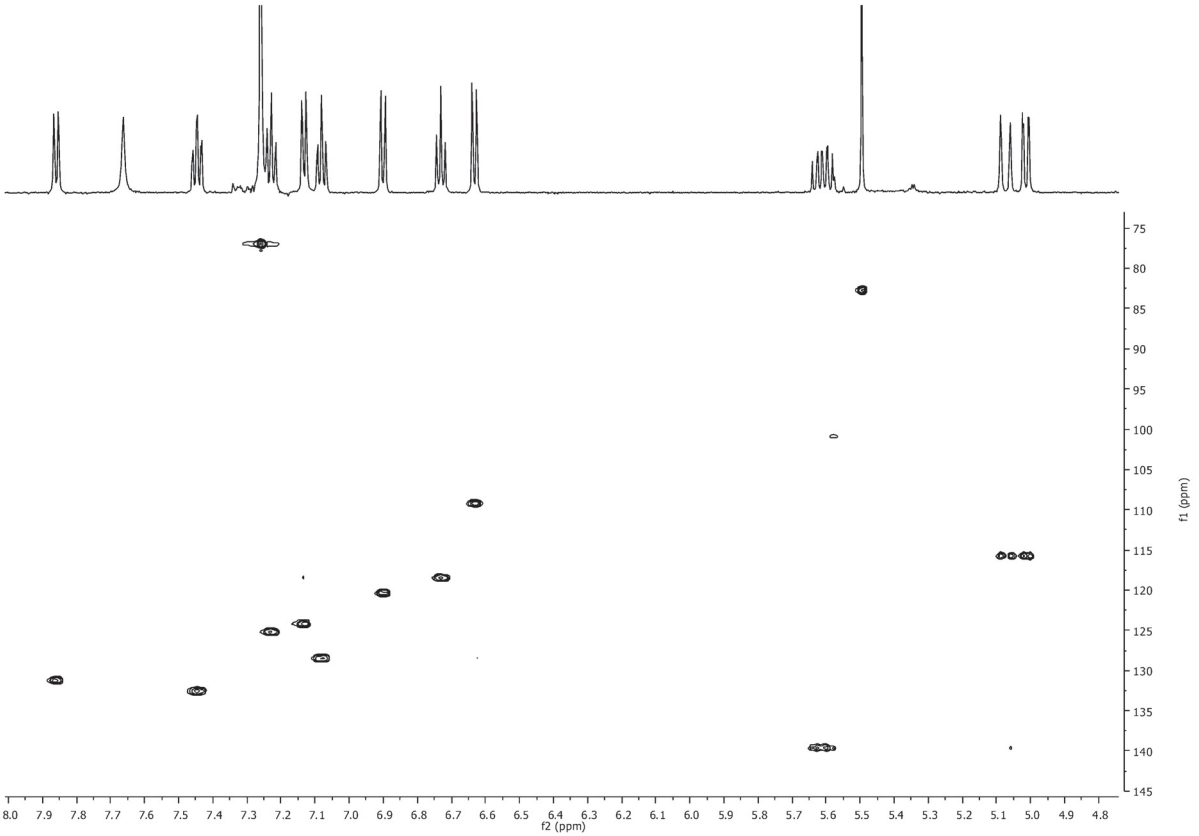


Figure S31. HSQC spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

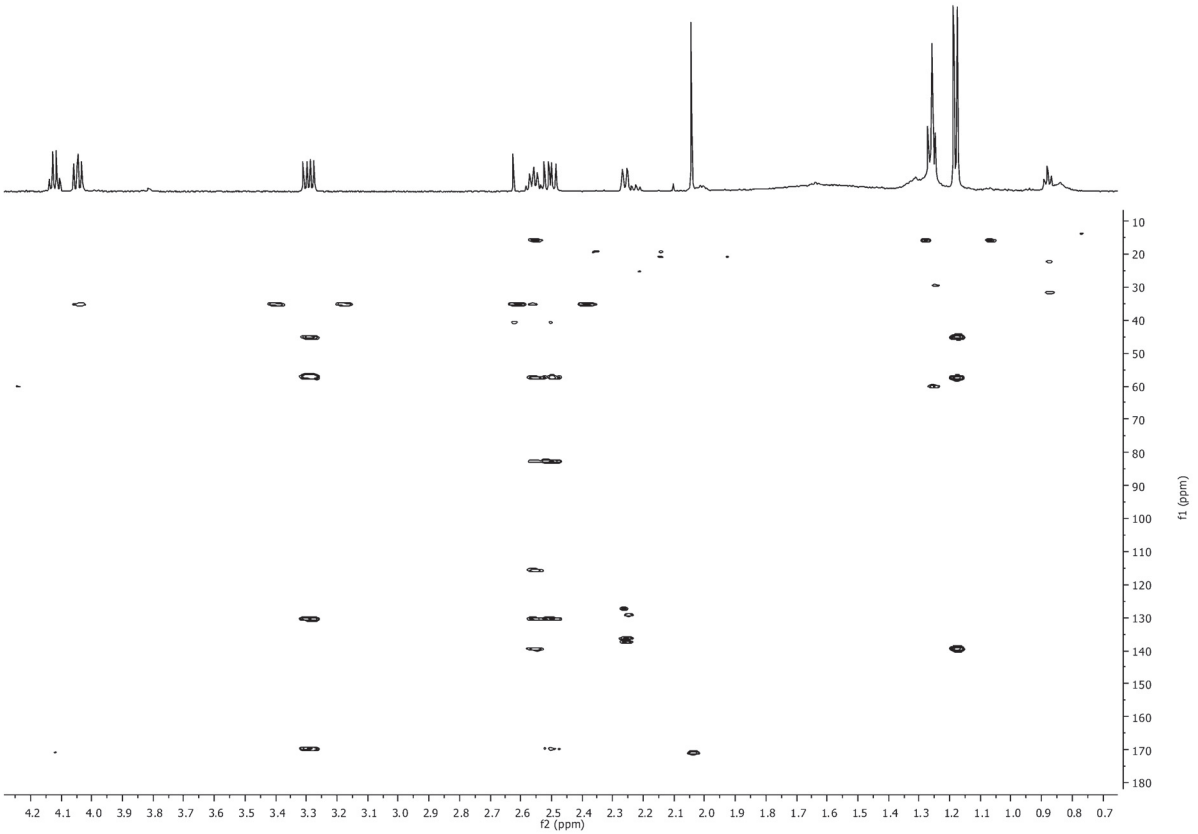


Figure S32. HMBC spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

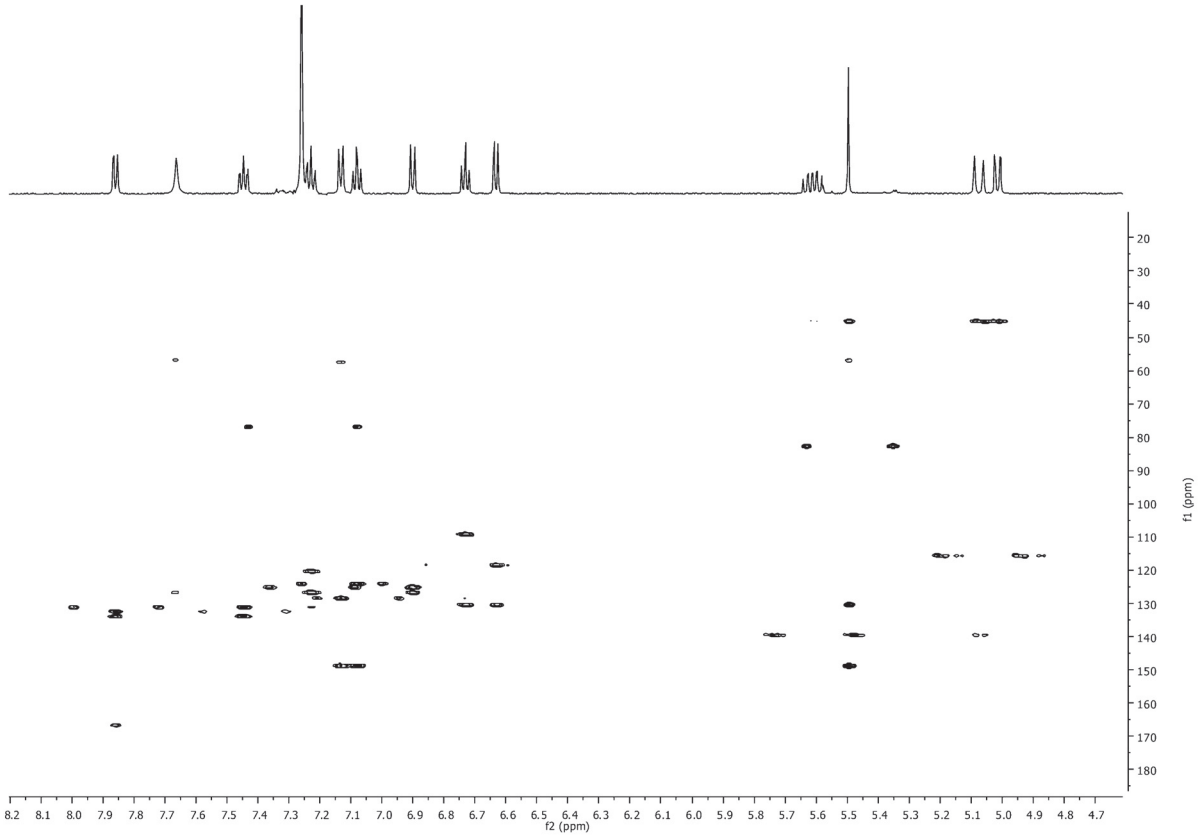


Figure S33. HMBC spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

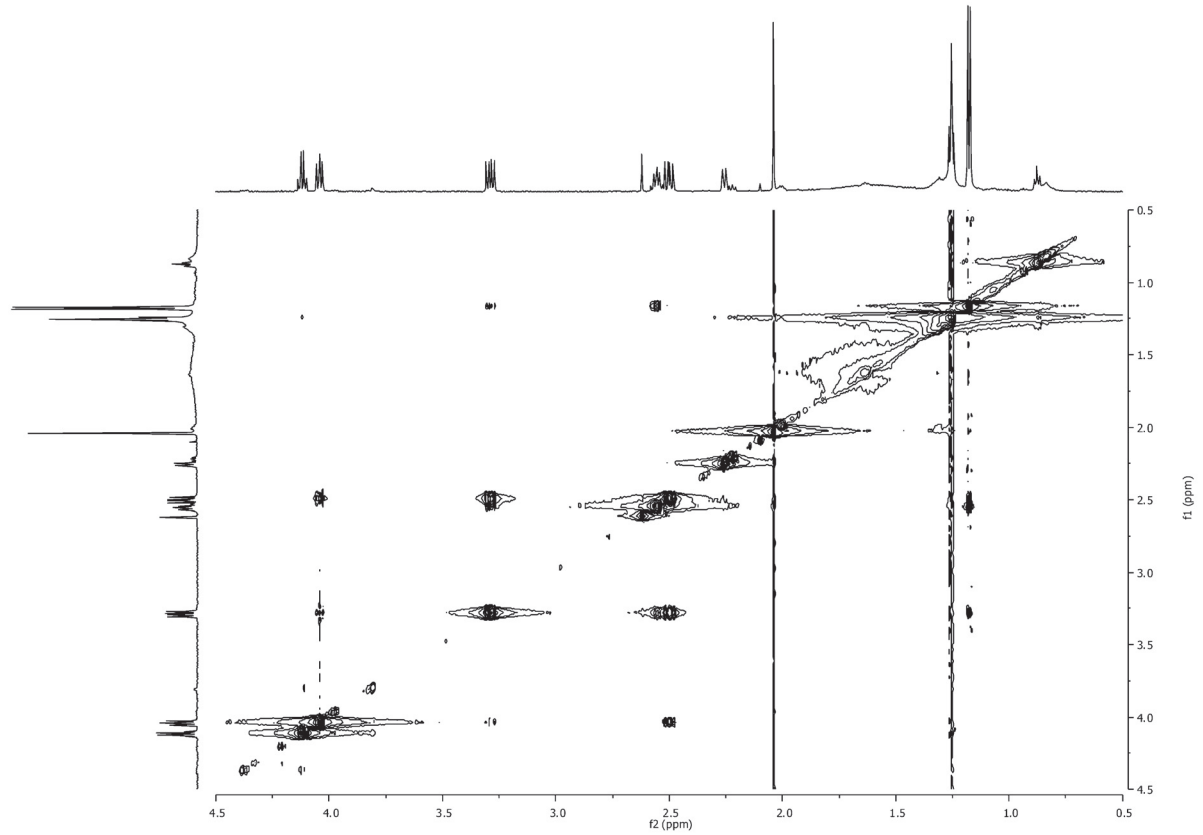


Figure S34. NOESY spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

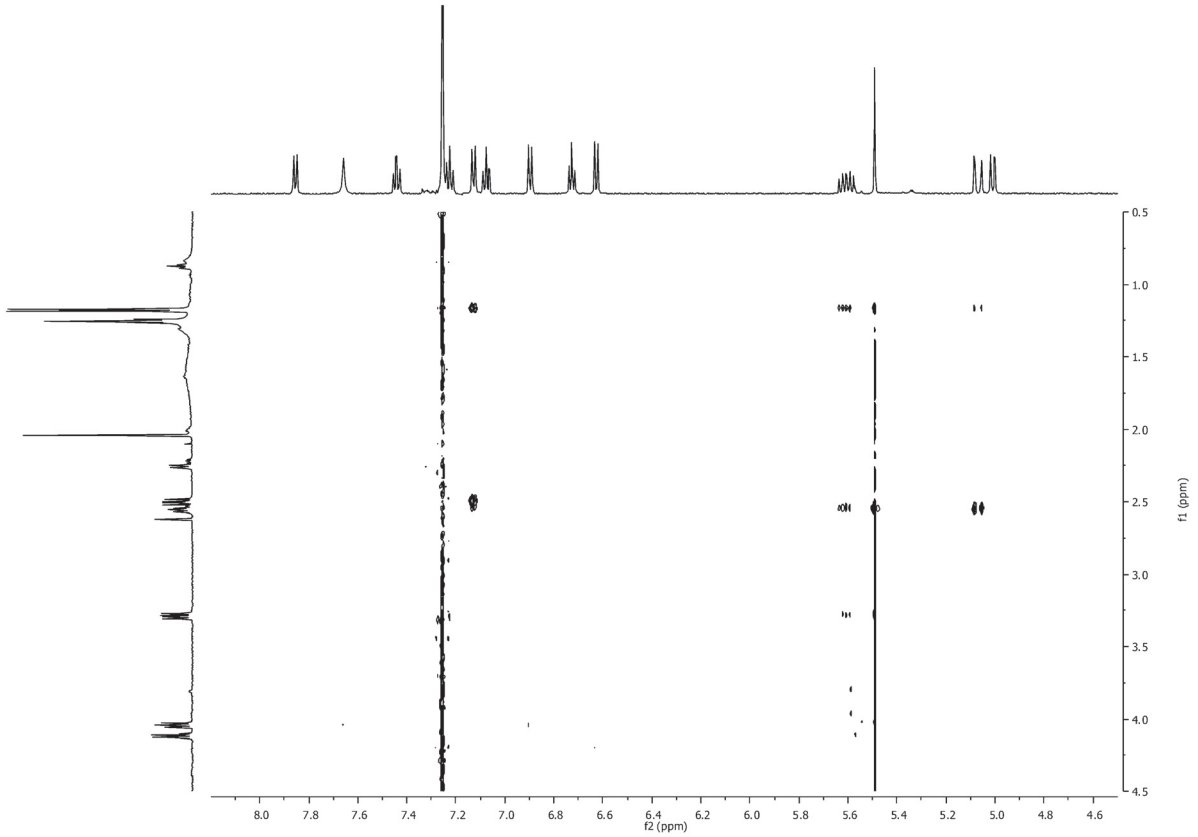


Figure S35. NOESY spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

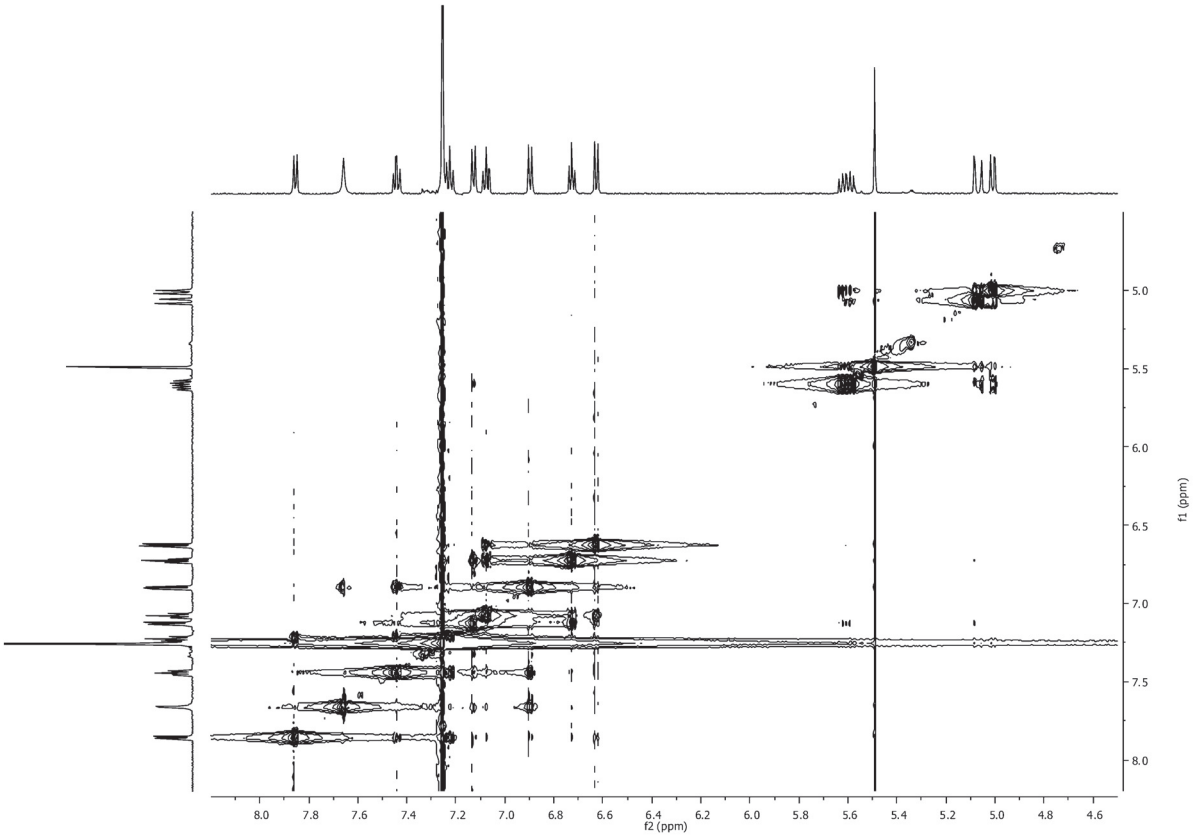


Figure S36. NOESY spectrum of C3α-(3'*S*-butenyl-)-(*R*)-benzodiazepindione (**I-1c**) in $CDCl_3$.

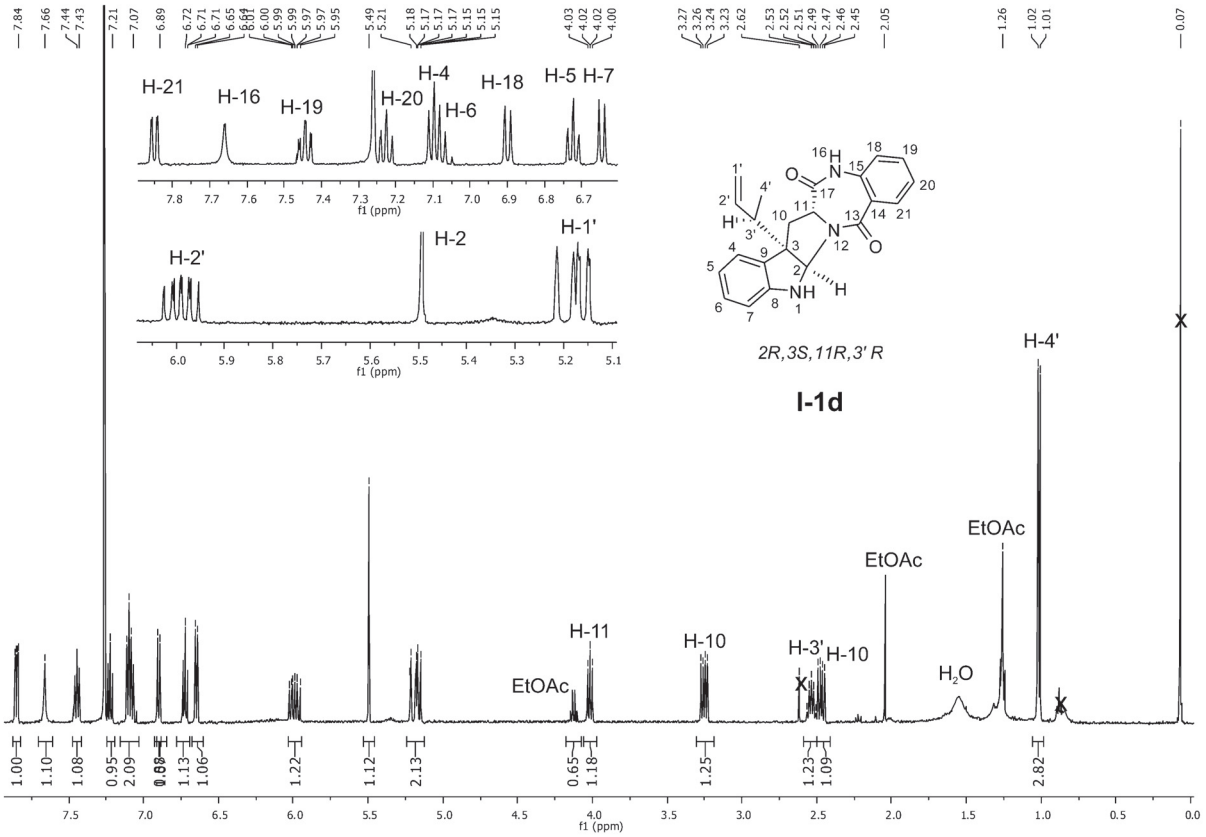


Figure S37. ^{1}H NMR spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

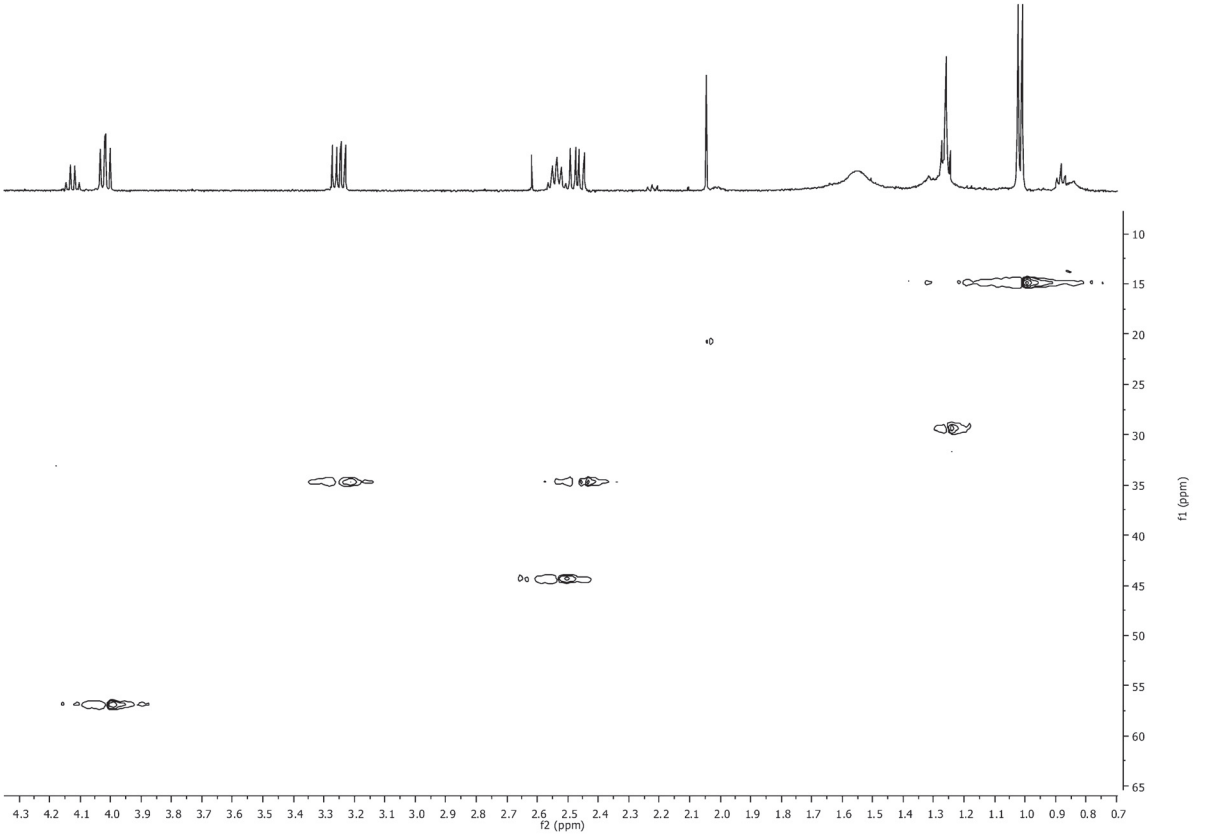


Figure S38. HSQC spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

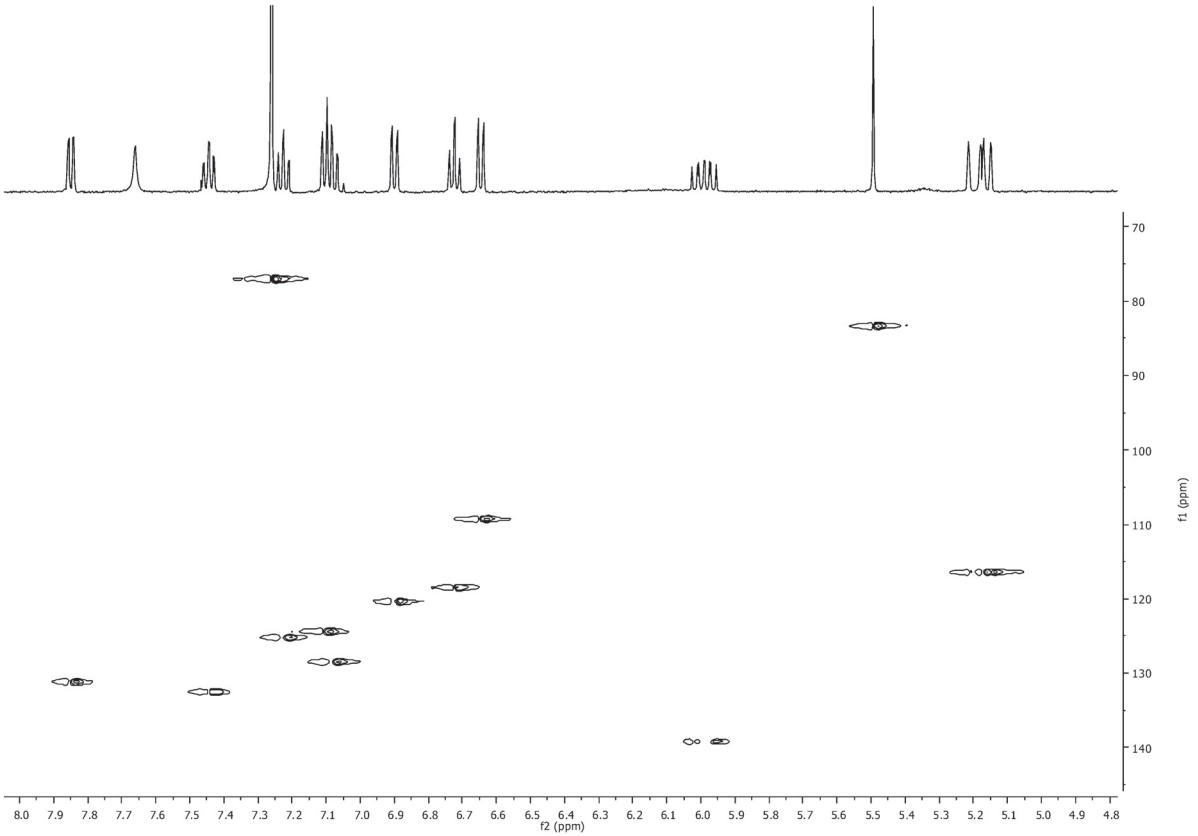


Figure S39. HSQC spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

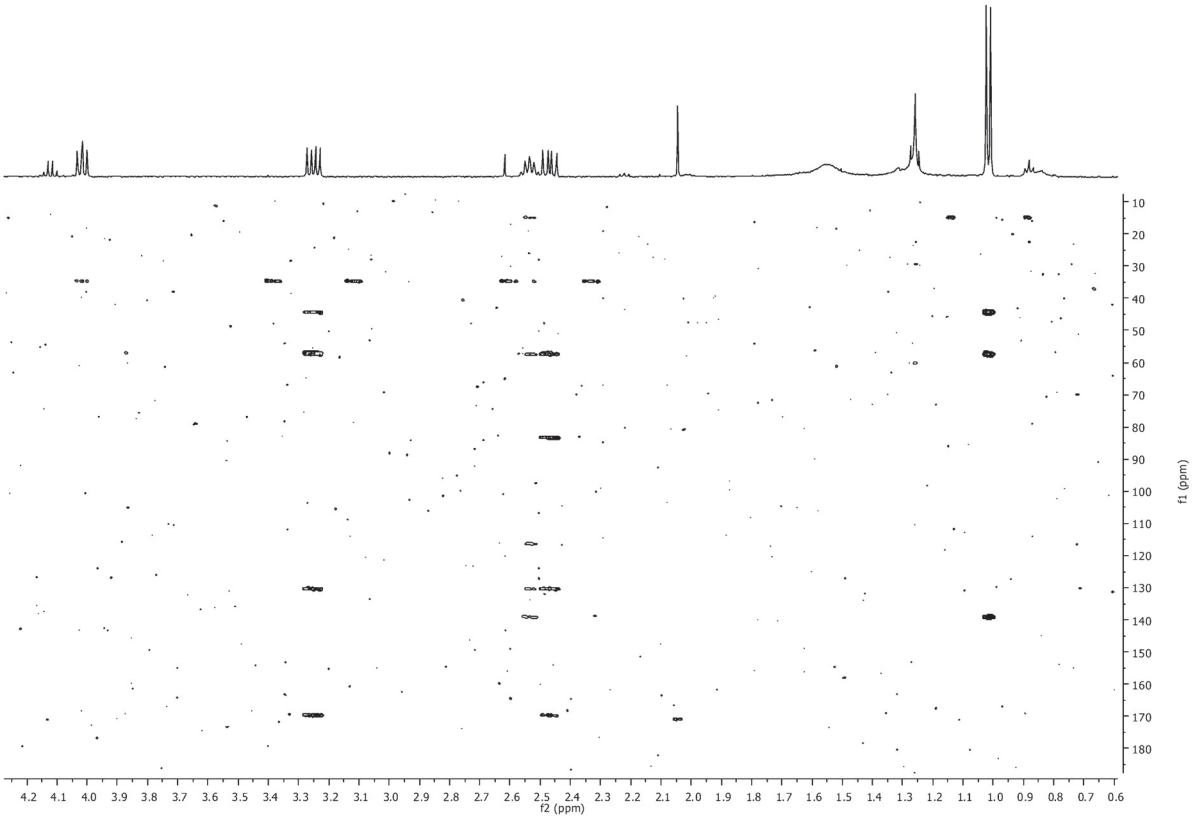


Figure S40. HMBC spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

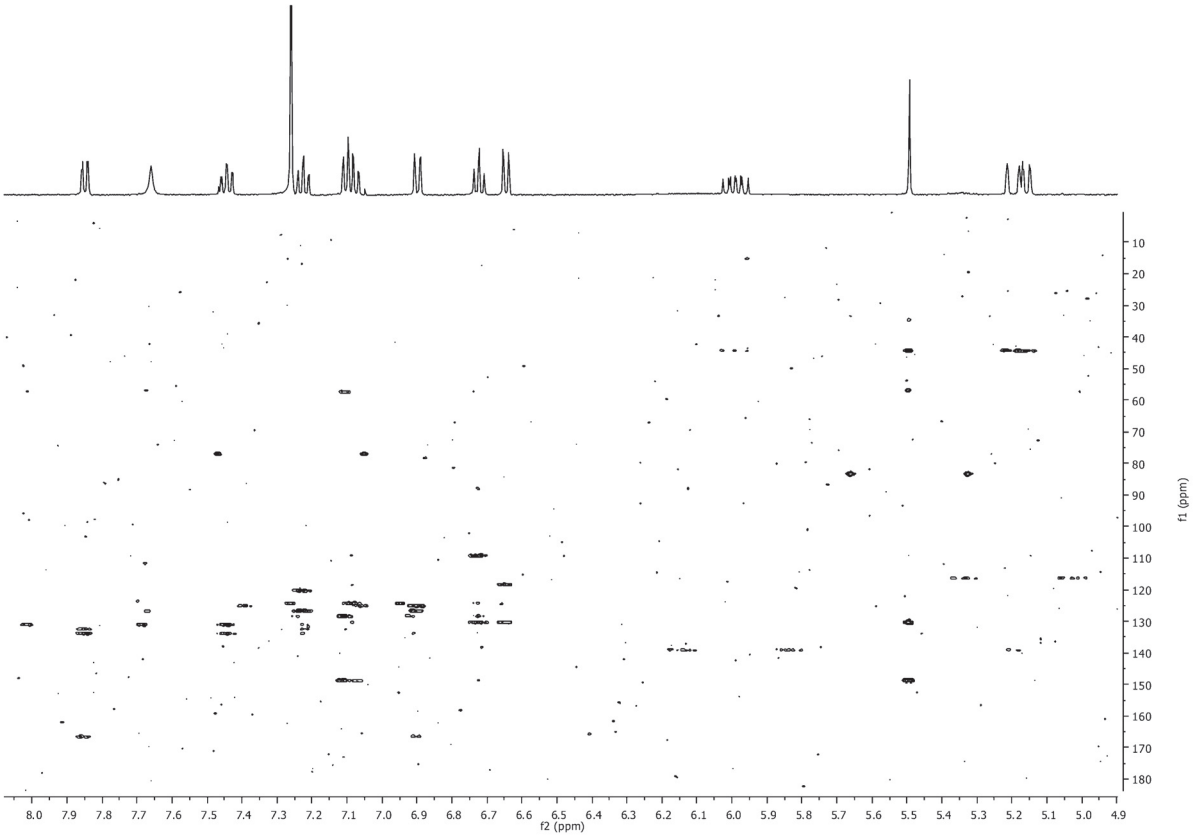


Figure S41. HMBC spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

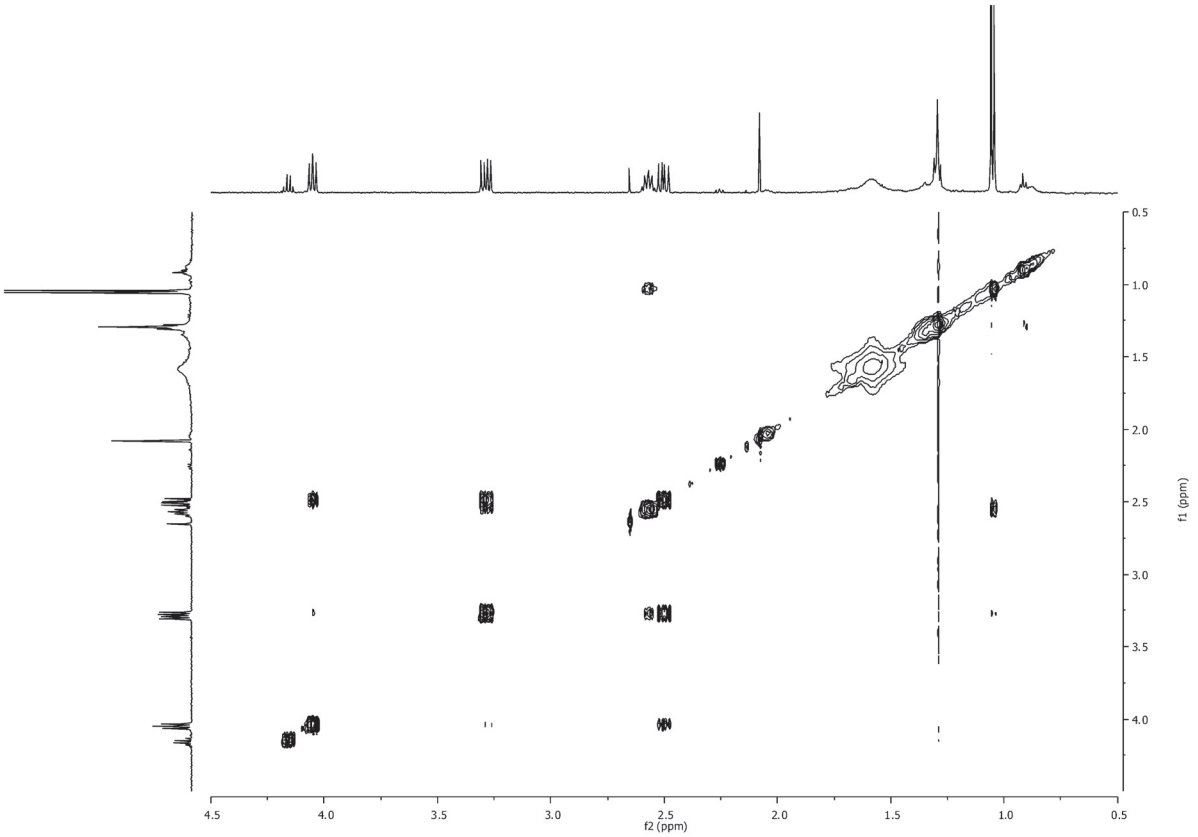


Figure S42. NOESY spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

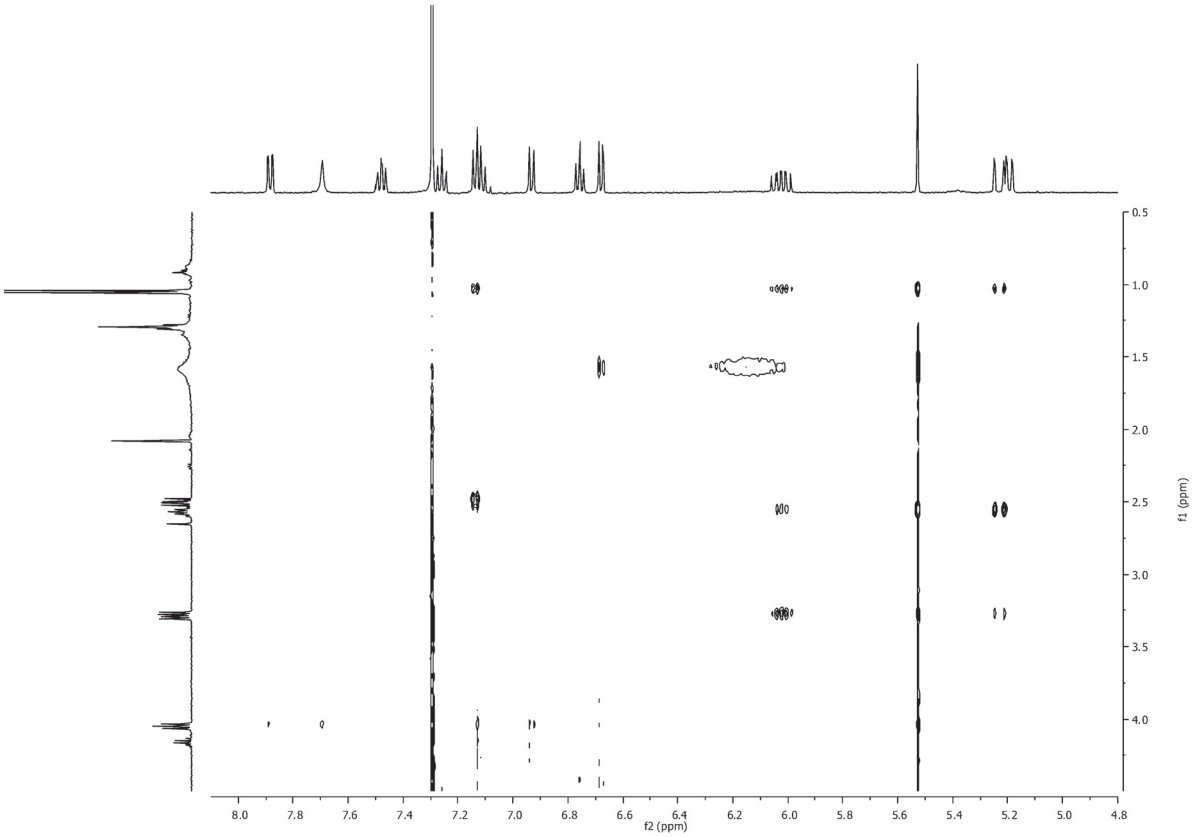


Figure S43. NOESY spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

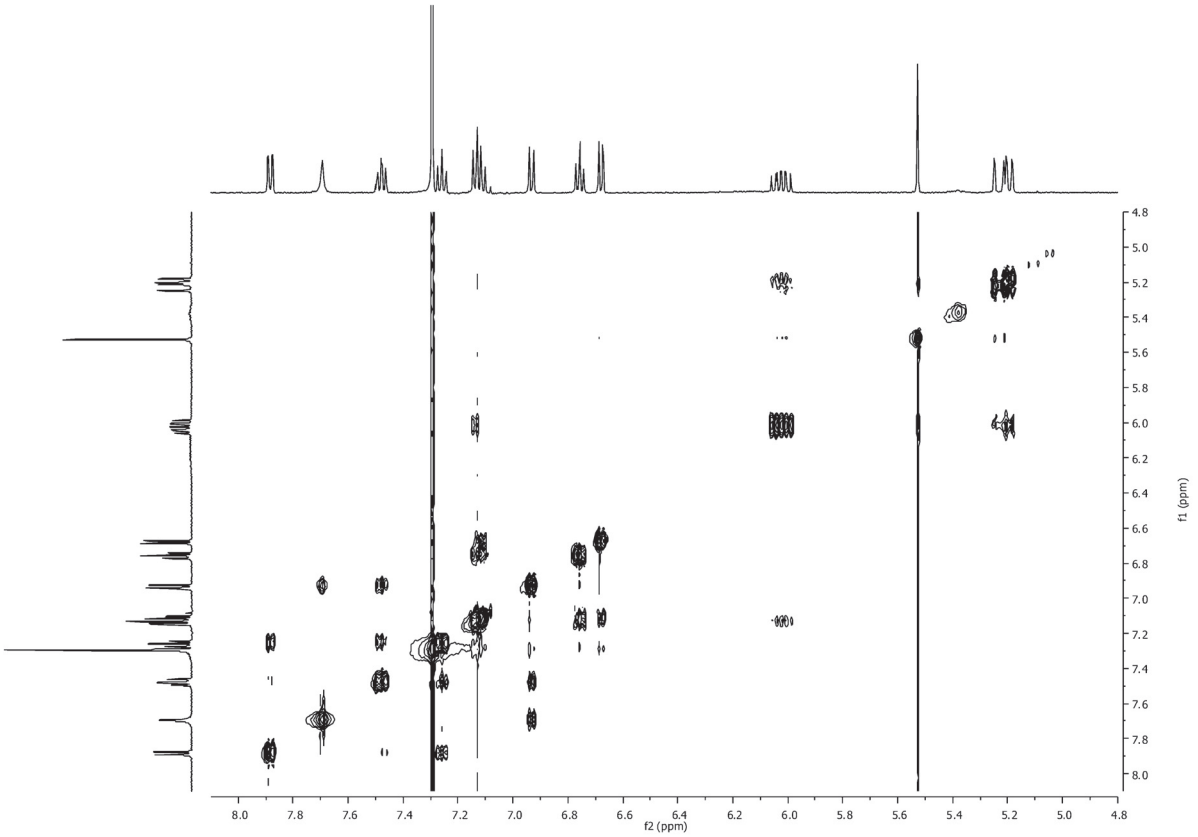


Figure S44. NOESY spectrum of C3α-(3'*R*-butenyl-)-(*R*)-benzodiazepindione (**I-1d**) in $CDCl_3$.

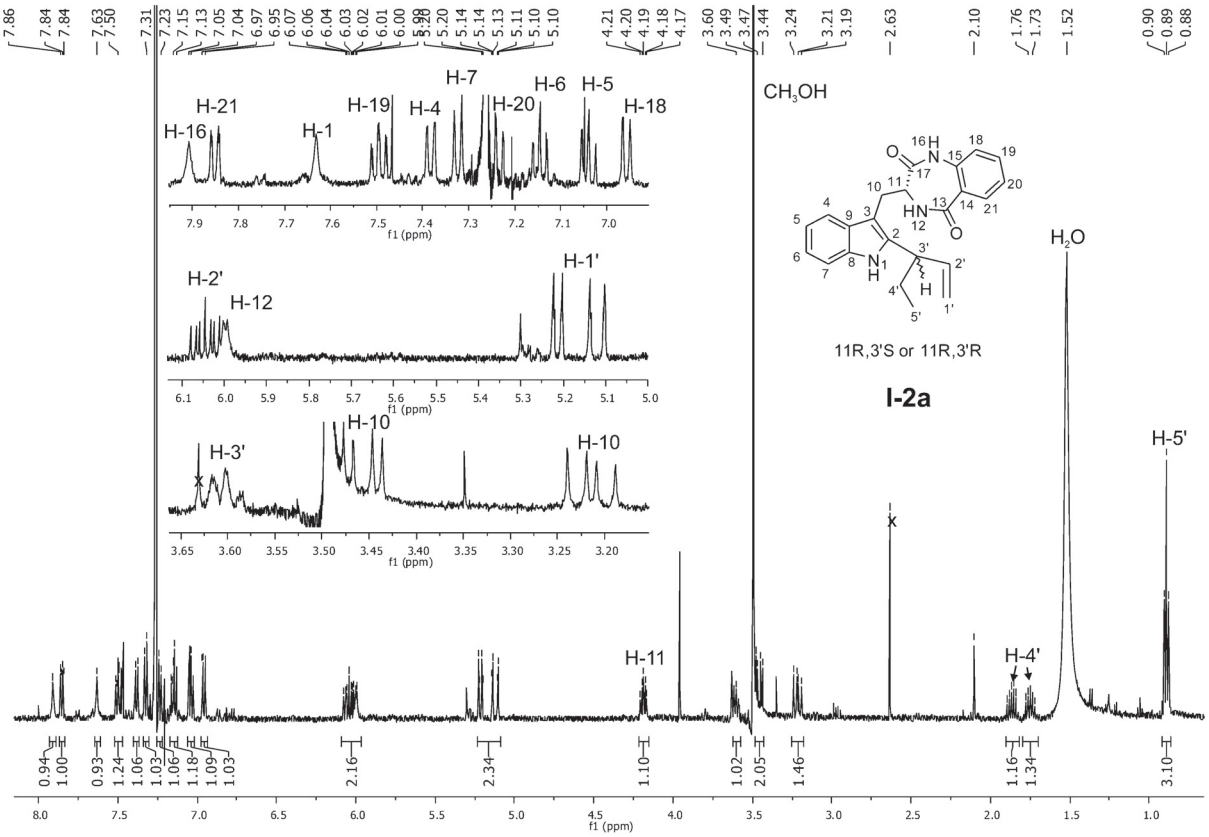


Figure S45. ^{1}H NMR spectrum of C2-(3'-pentenyl-)-(*R*)-benzodiazepindione (**I-2a**) in $CDCl_3$.

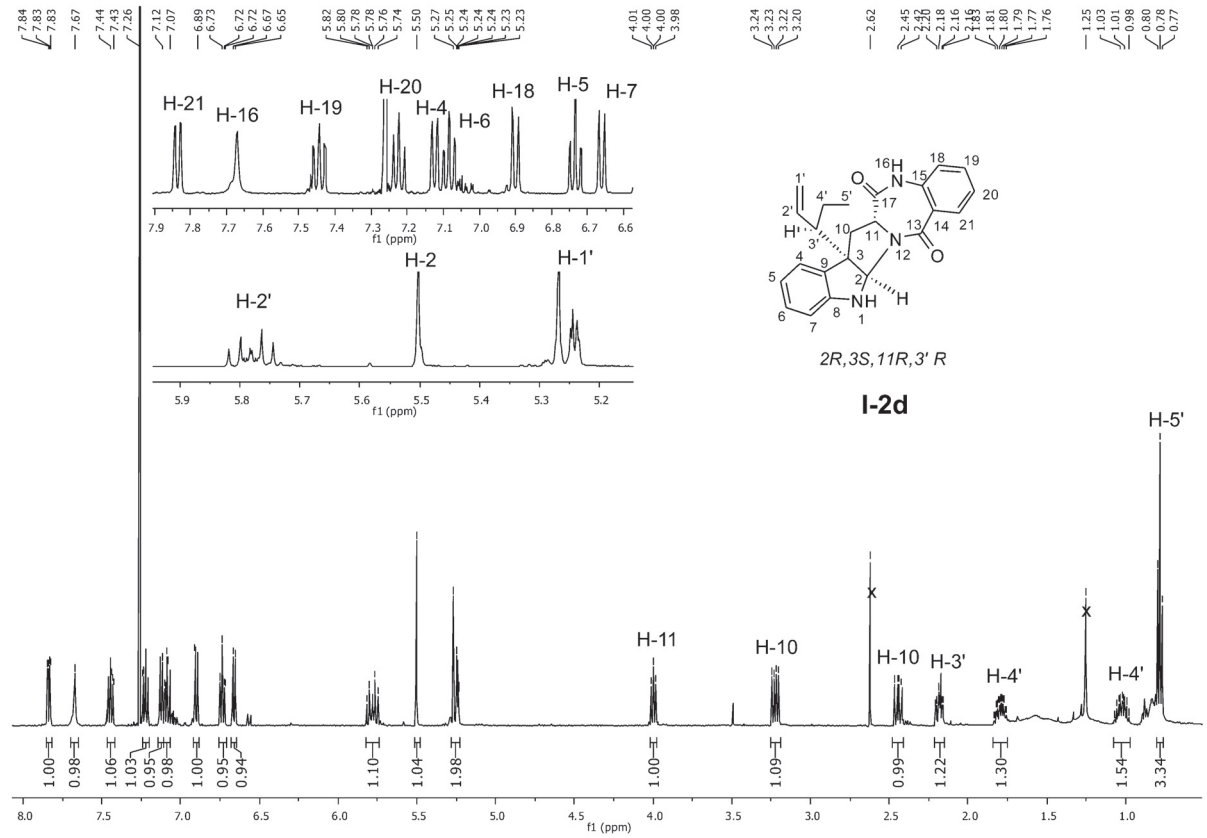


Figure S46. ^{1}H NMR spectrum of C3α-(3'*R*-pentenyl-)-(*R*)-benzodiazepindione (**I-2d**) in $CDCl_3$.

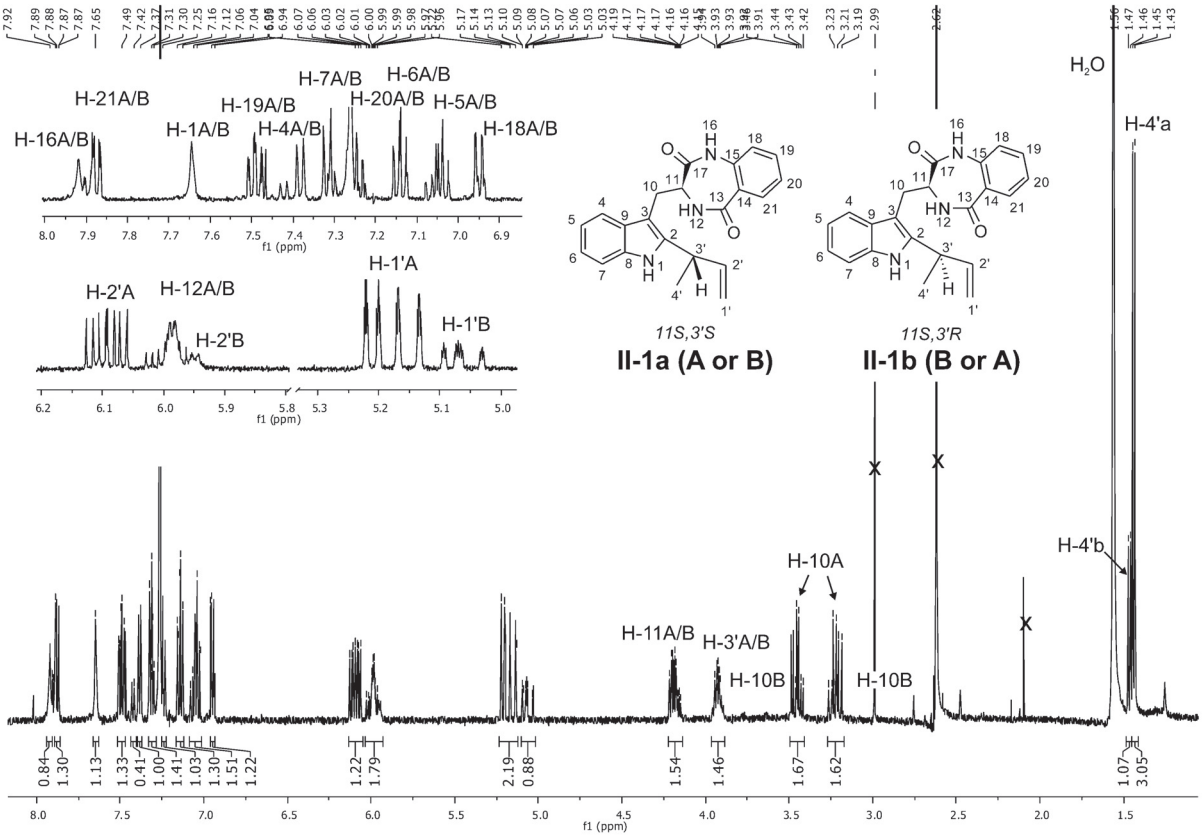


Figure S47. ^{1}H NMR spectrum of mixture C2-(3'*R*-butenyl-)-(*S*)-benzodiazepindione and C2-(3'*S*-butenyl-)-(*S*)-benzodiazepindione (**II-1a+b**) in $CDCl_3$.

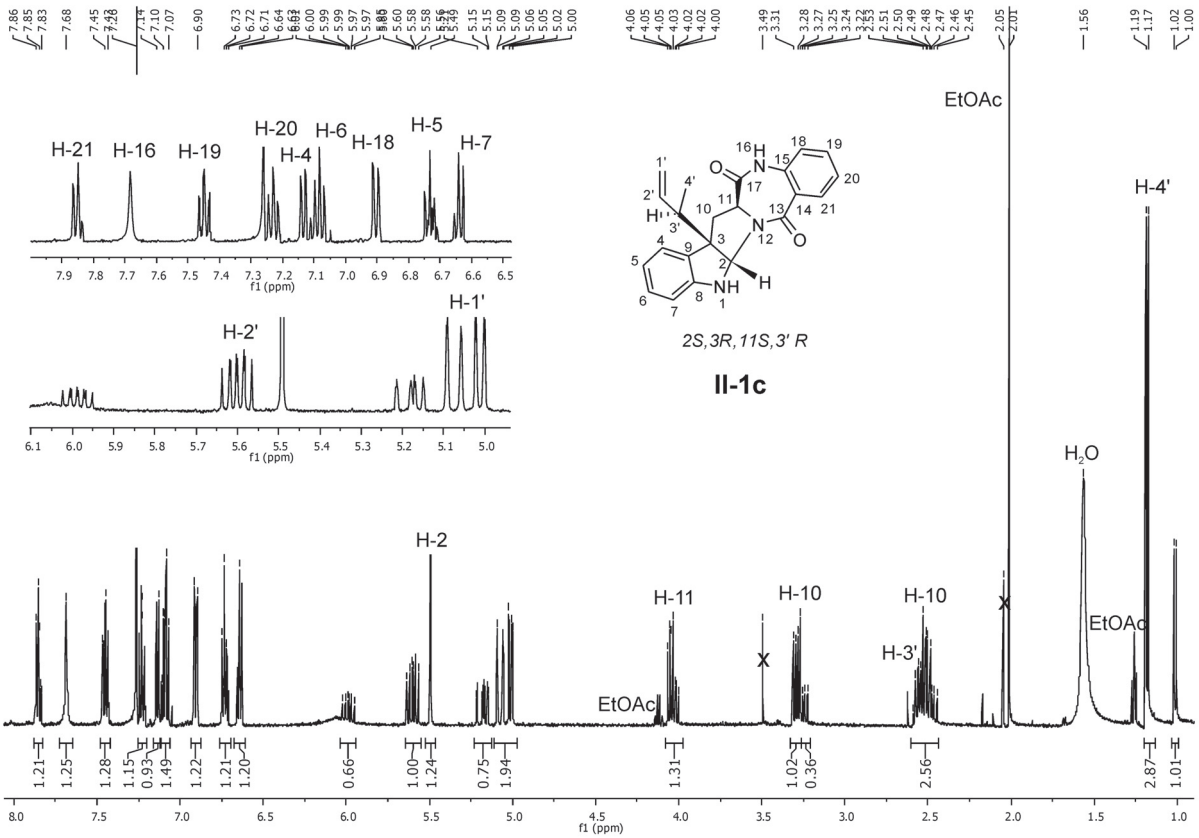


Figure S48. ^{1}H NMR spectrum of C3β-(3'*R*-butenyl-)-(*S*)-benzodiazepindione (**II-1c**) in $CDCl_3$.

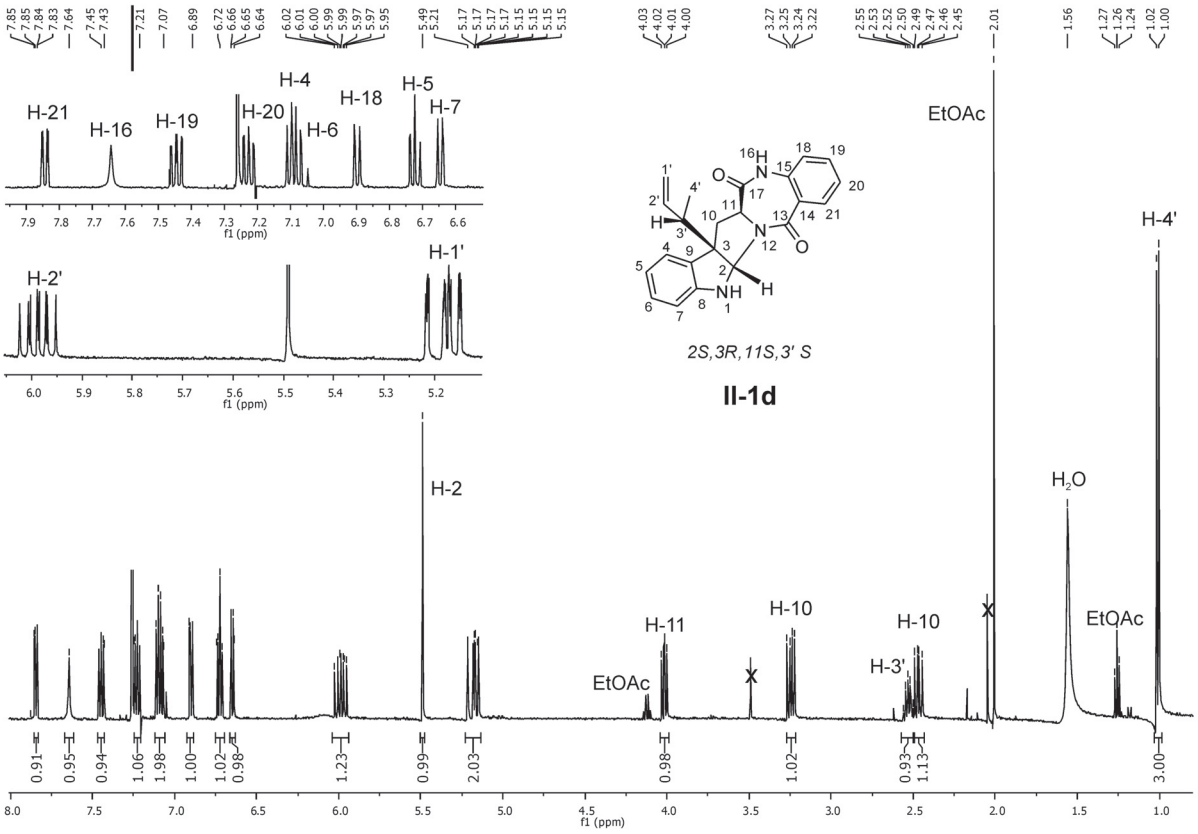


Figure S49. ^{1}H NMR spectrum of C3β-(3'*S*-butenyl-)-(*S*)-benzodiazepindione (**II-1d**) in $CDCl_3$.

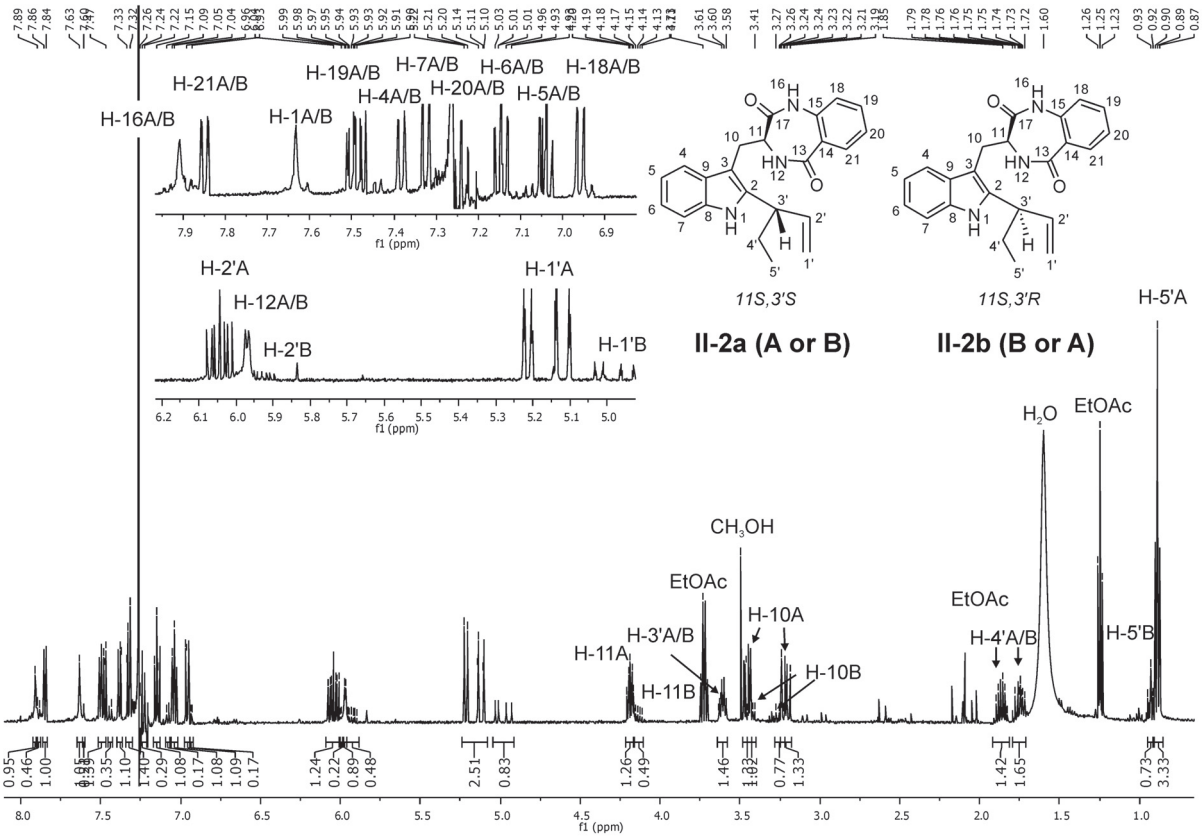


Figure S50. ^{1}H NMR spectrum of mixture C2-(3'*R*-pentenyl-)-(*S*)-benzodiazepindione and C2-(3'*S*-pentenyl-)-(*S*)-benzodiazepindione (**II-2a+2b**) in $CDCl_3$.

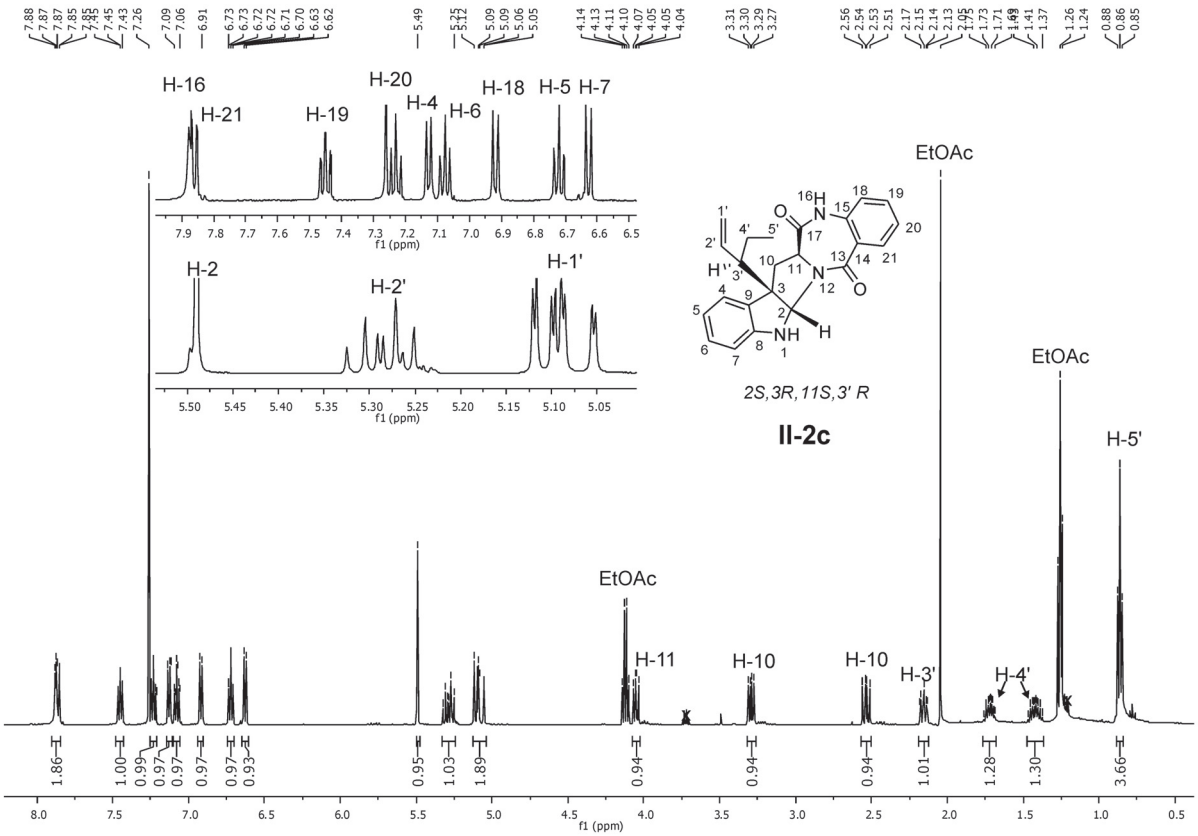


Figure S51. ^{1}H NMR spectrum of C3β-(3'*R*-pentenyl-)-(*S*)-benzodiazepindione (**II-2c**) in $CDCl_3$.

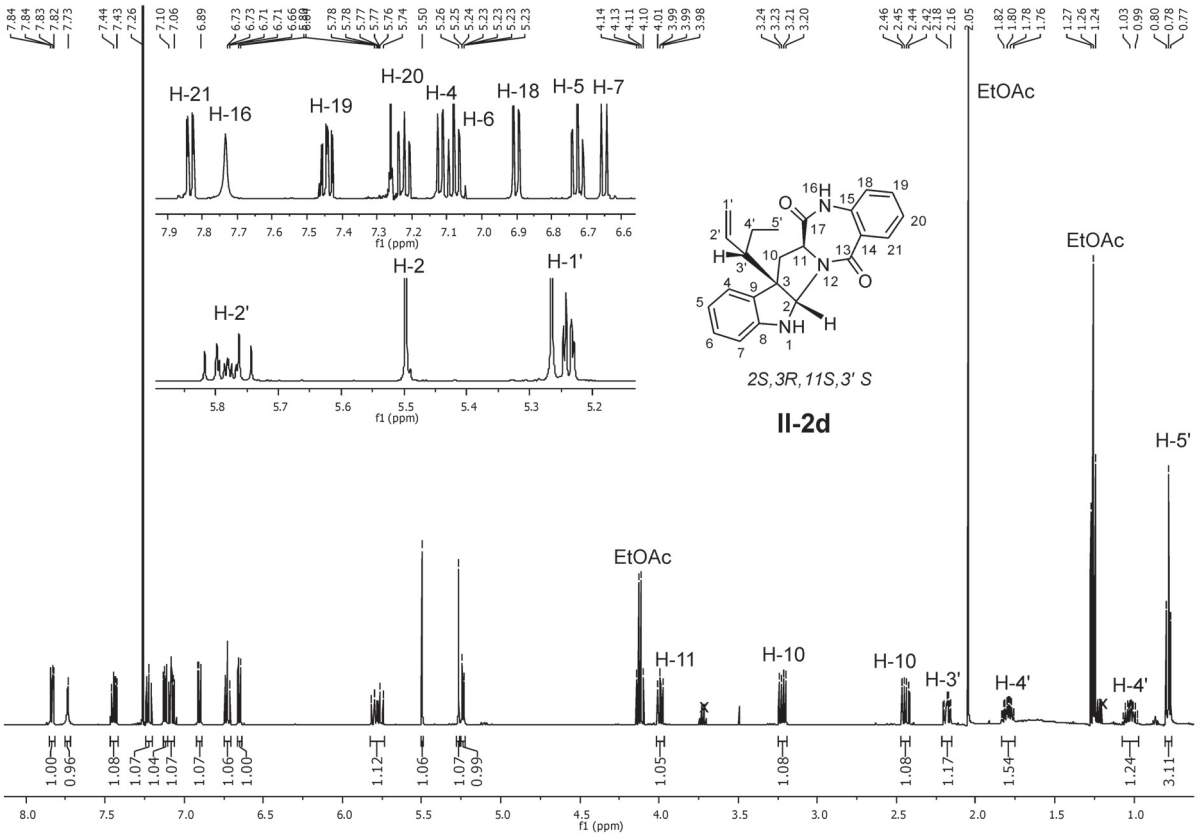


Figure S52. ^{1}H NMR spectrum of C3β-(3'*S*-pentenyl-)-(*S*)-benzodiazepindione (**II-2d**) in $CDCl_3$.

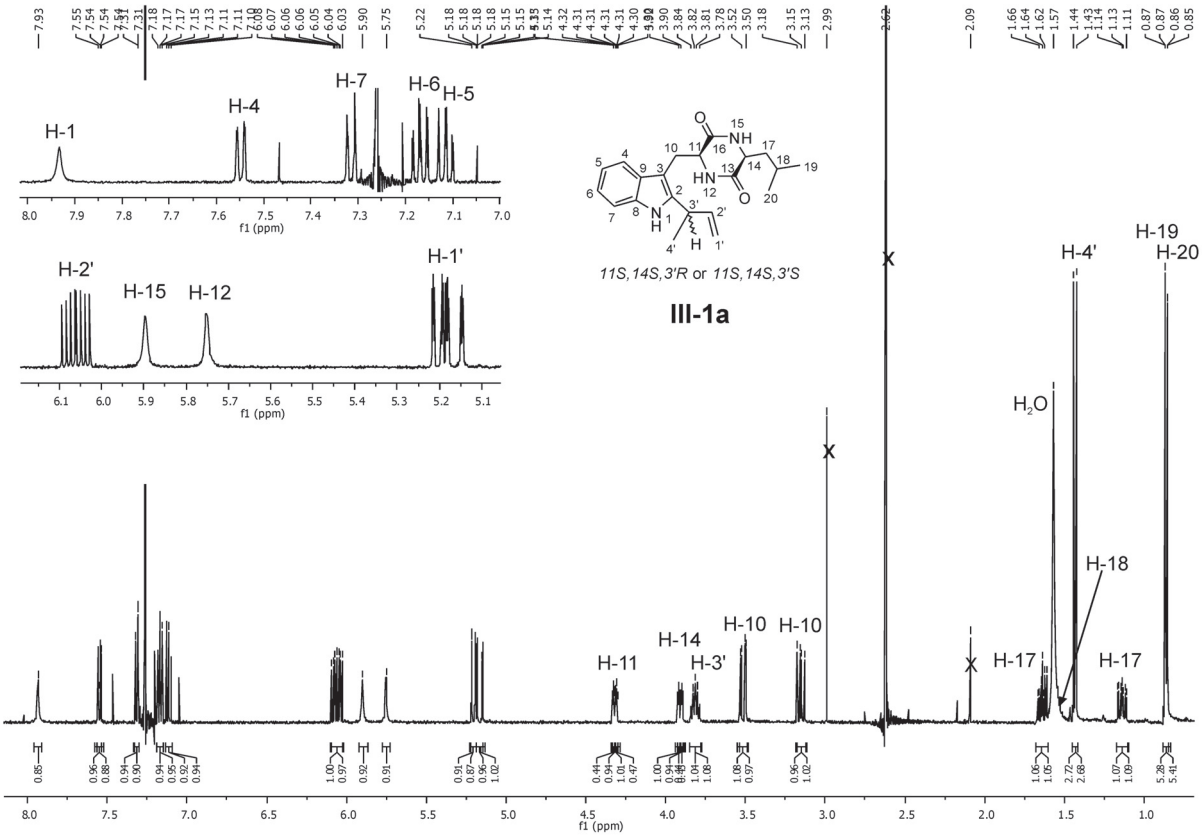


Figure S53. ^{1}H NMR spectrum of *cyclo*-C2-(3'-butenyl-)-L-Trp-L-Leu (**III-1a**) in $CDCl_3$.

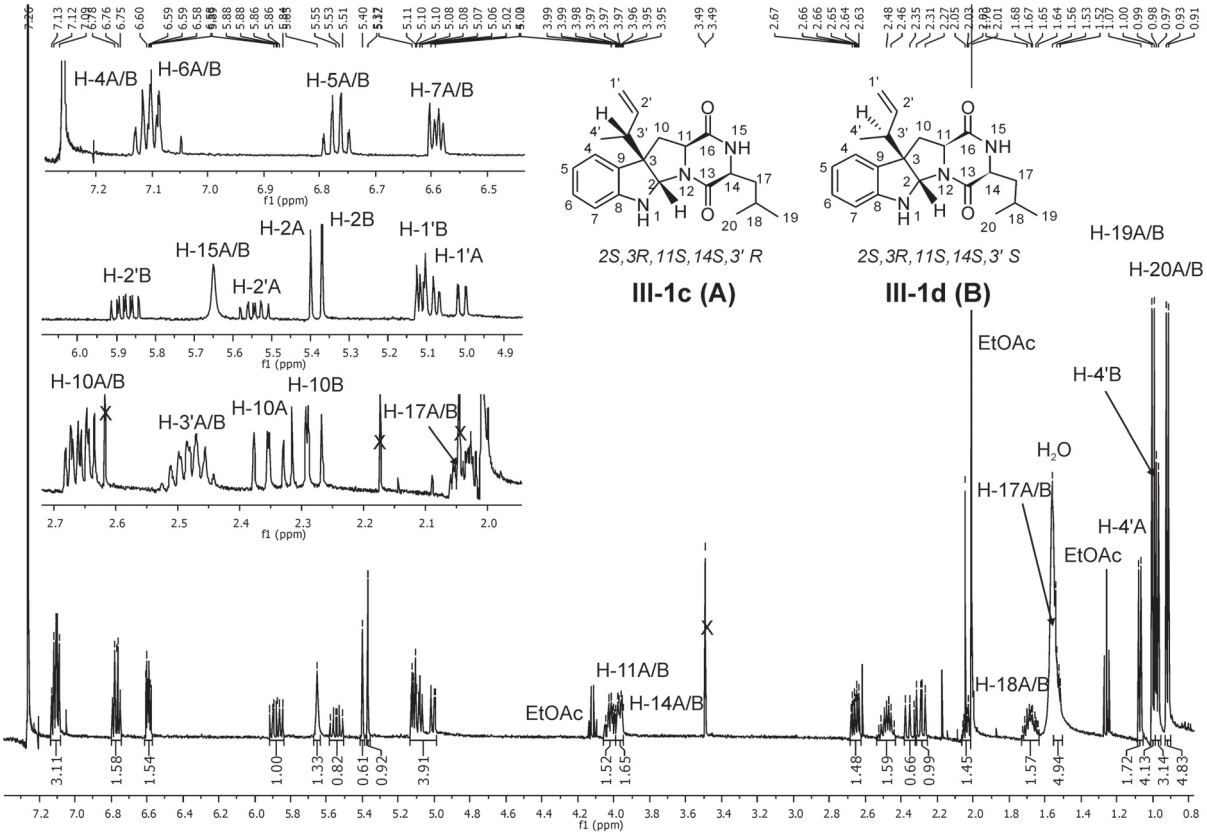


Figure S54. ^{1}H NMR spectrum of mixture *cyclo*-C3β-(3'*R*-butenyl-)-L-Trp-L-Leu (**III-1c**) and *cyclo*-C3β-(3'*S*-butenyl-)-L-Trp-L-Leu (**III-1d**) in $CDCl_3$ before separation leading to spectra Figure S55 and S56.

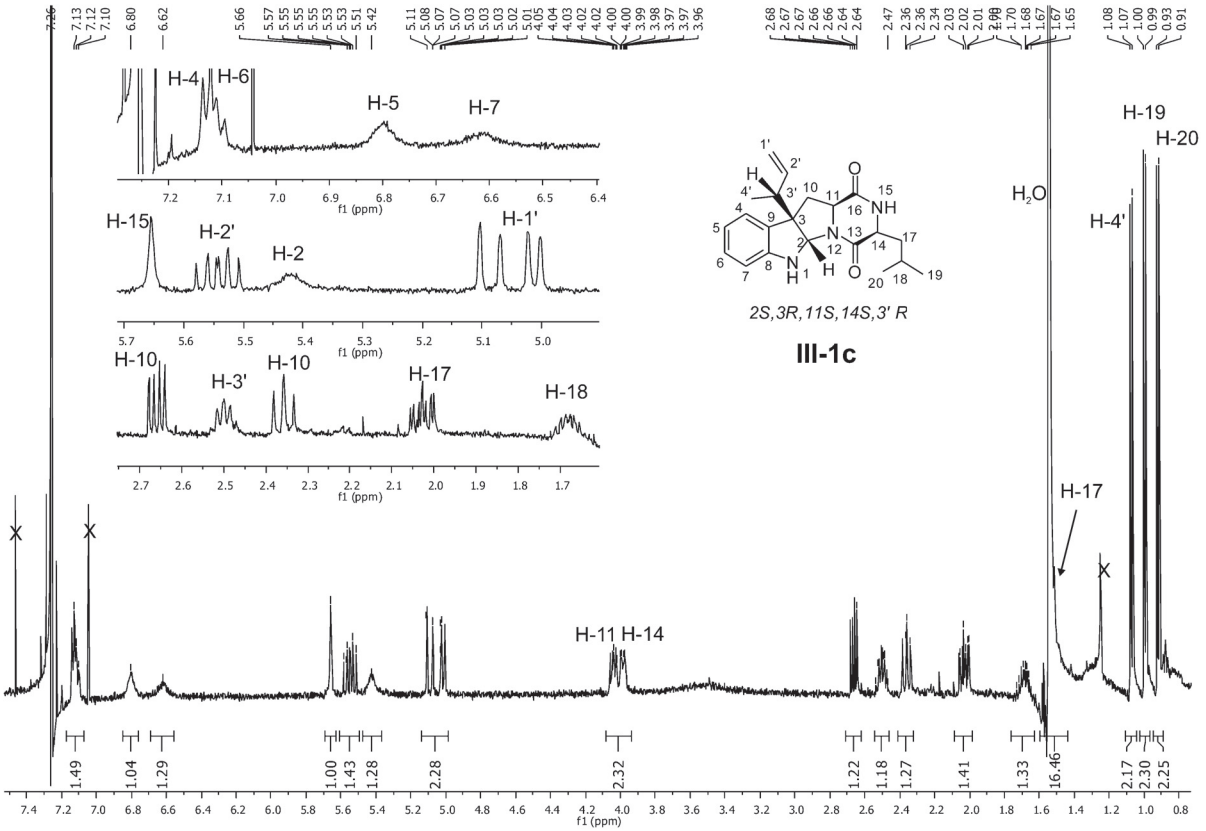


Figure S55. ^{1}H NMR spectrum of *cyclo*-C3β-(3'*R*-butenyl-)-L-Trp-L-Leu (**III-1c**) in $CDCl_3$ after separation of mixture shown in Figure S54.

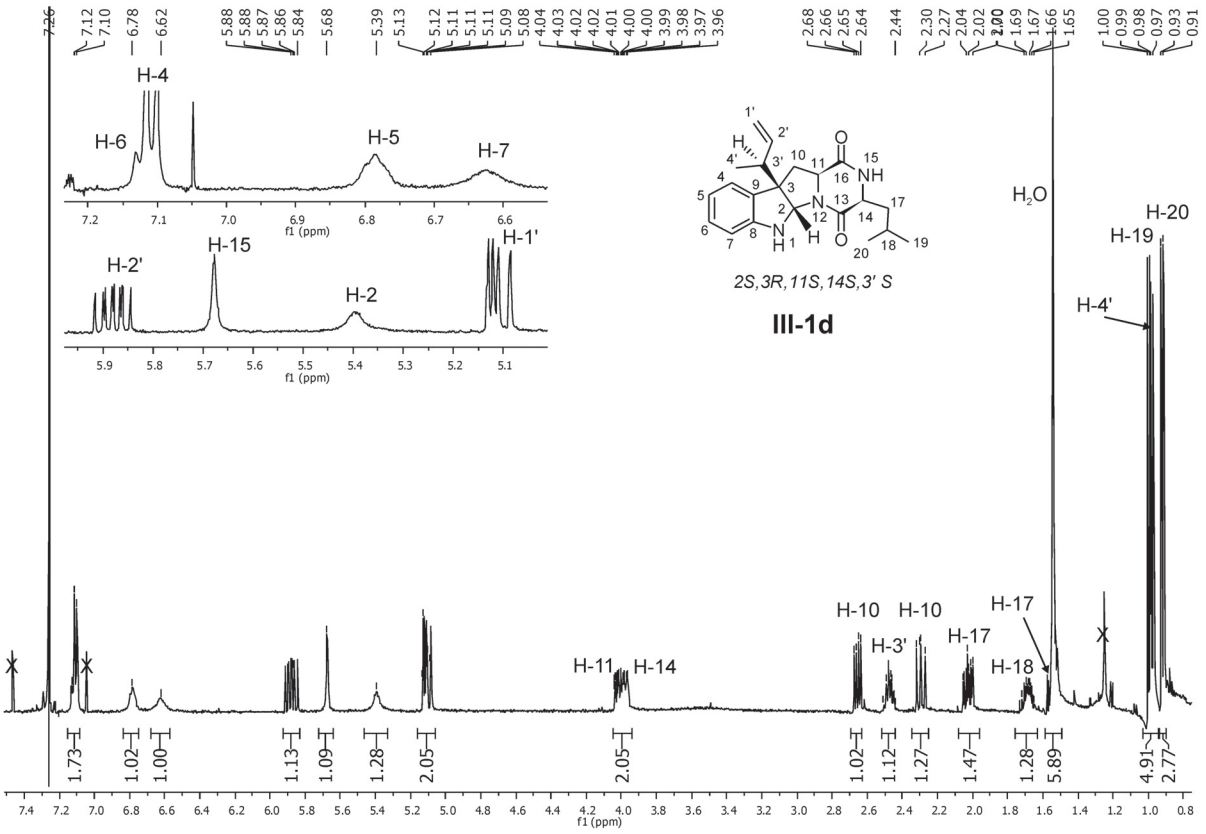


Figure S56. ^{1}H NMR spectrum of *cyclo*-C3β-(3'*S*-butenyl-)-L-Trp-L-Leu (**III-1d**) in $CDCl_3$ after separation of mixture shown in Figure S54.

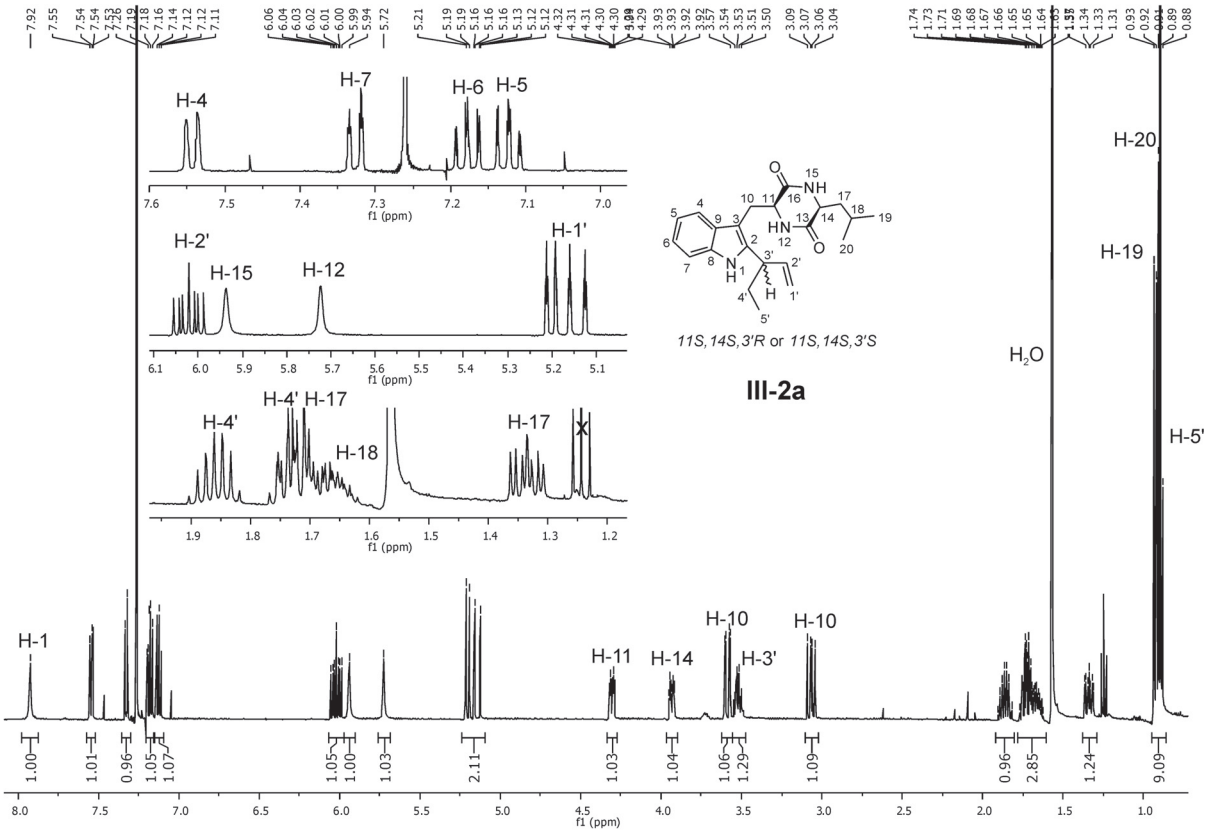


Figure S57. ^{1}H NMR spectrum of *cyclo*-C2-(3'-pentenyl-)-L-Trp-L-Leu (**III-2a**) in $CDCl_3$.

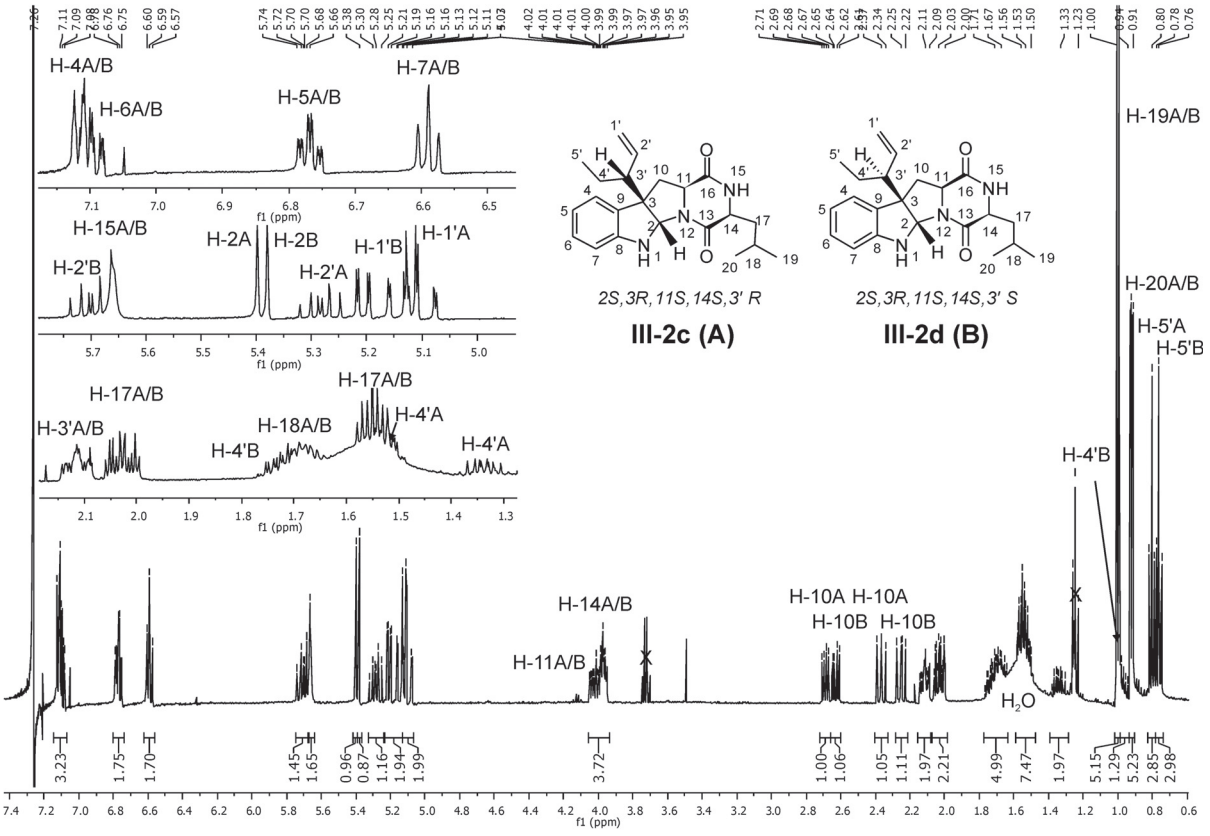


Figure S58. ^{1}H NMR spectrum of mixture *cyclo*-C3β-(3'*R*-pentenyl-)-L-Trp-L-Leu (**III-2c**) and *cyclo*-C3β-(3'*S*-pentenyl-)-L-Trp-L-Leu (**III-2d**) in $CDCl_3$ before separation leading to spectra Figure S59 and S60.

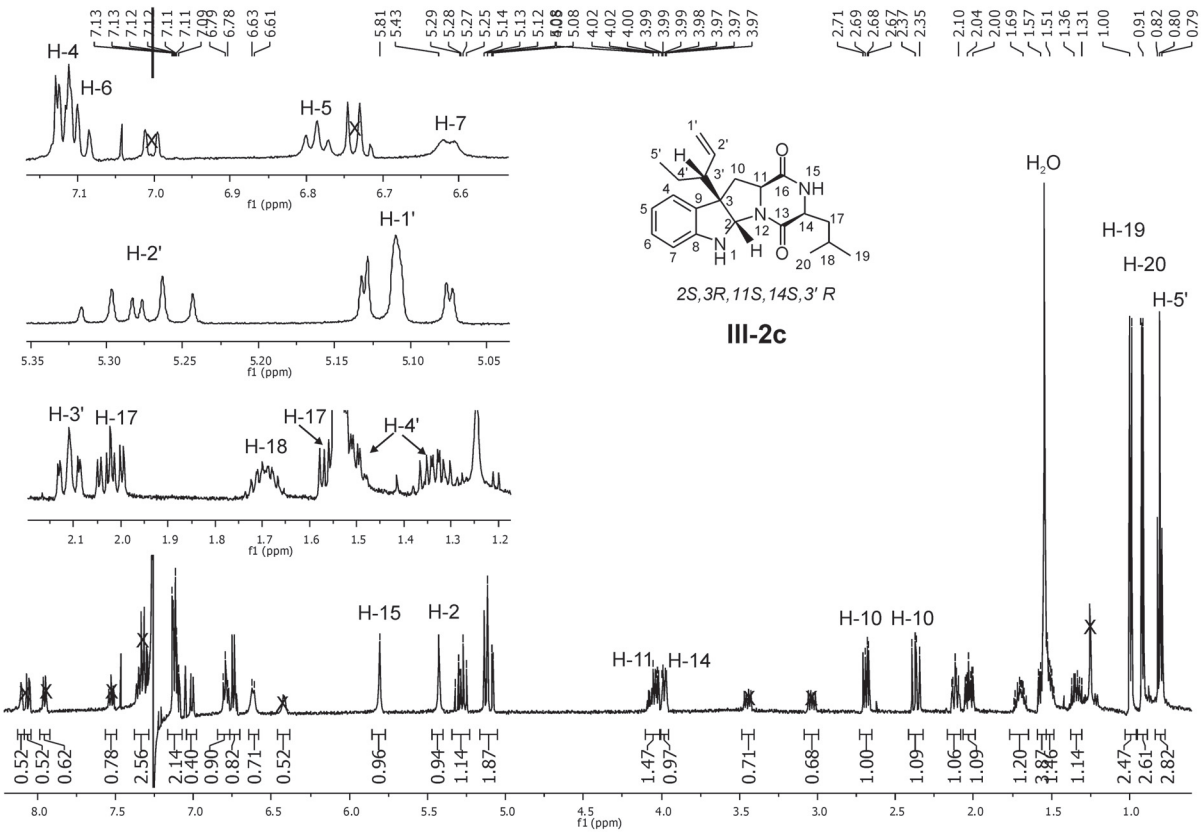


Figure S59. ^{1}H NMR spectrum of *cyclo*-C3β-(3'*R*-pentenyl-)-L-Trp-L-Leu (**III-2c**) in $CDCl_3$ after separation of mixture shown in Figure S58.

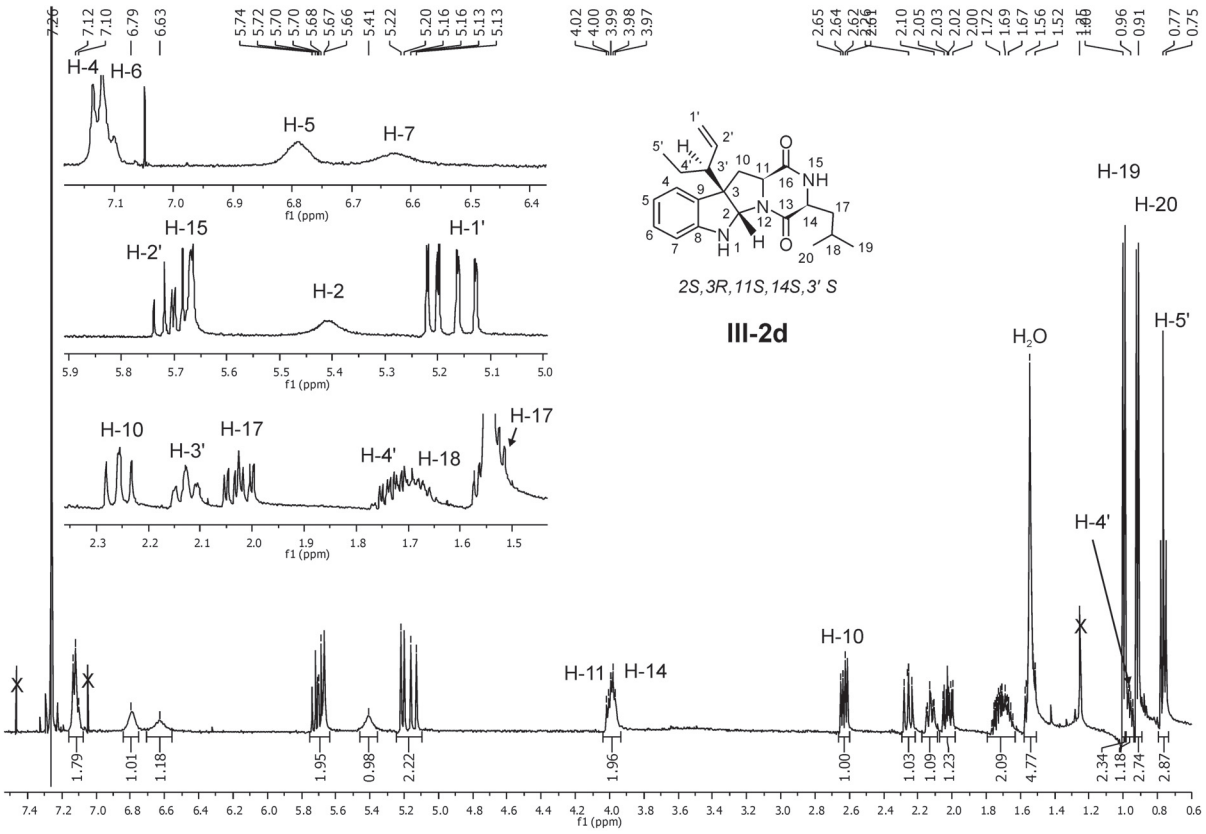


Figure S60. ^{1}H NMR spectrum of *cyclo*-C3β-(3'*S*-pentenyl-)-L-Trp-L-Leu (**III-2d**) in $CDCl_3$ after separation of mixture shown in Figure S58.

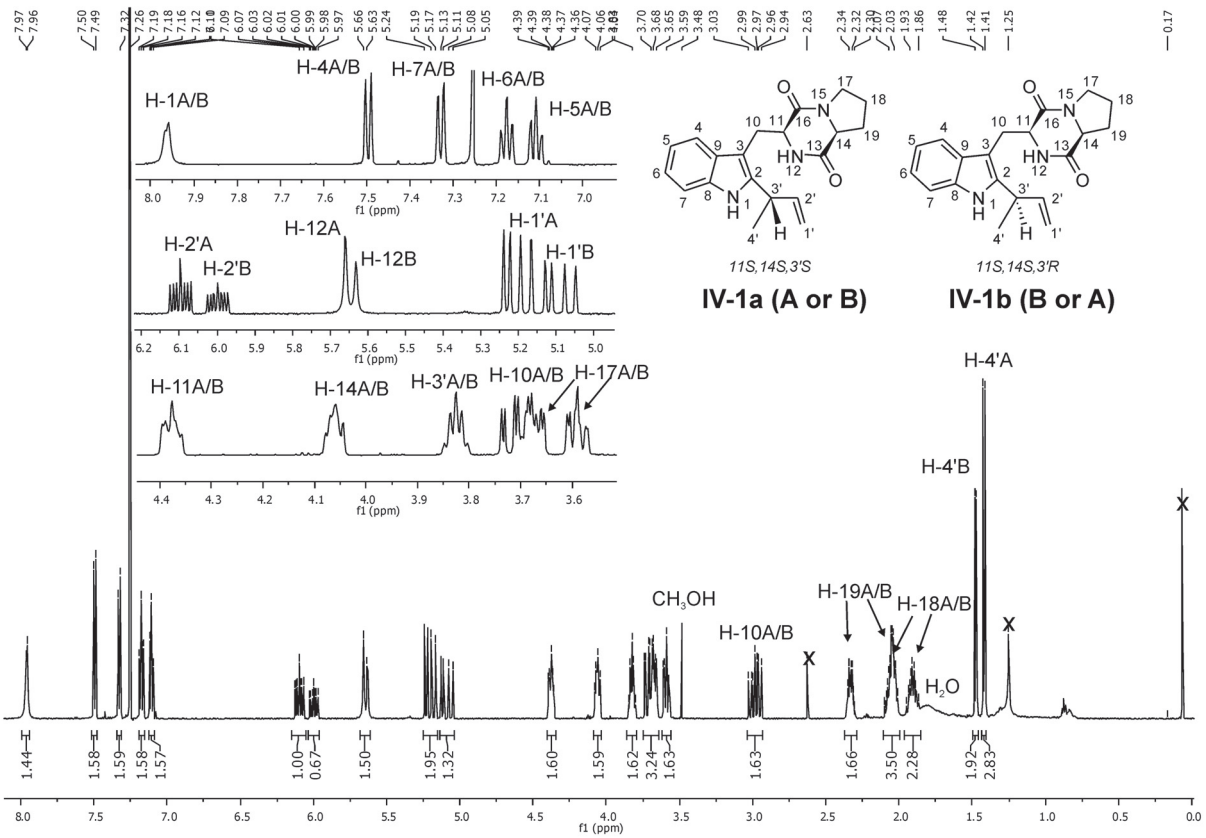


Figure S61. ^{1}H NMR spectrum of mixture *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

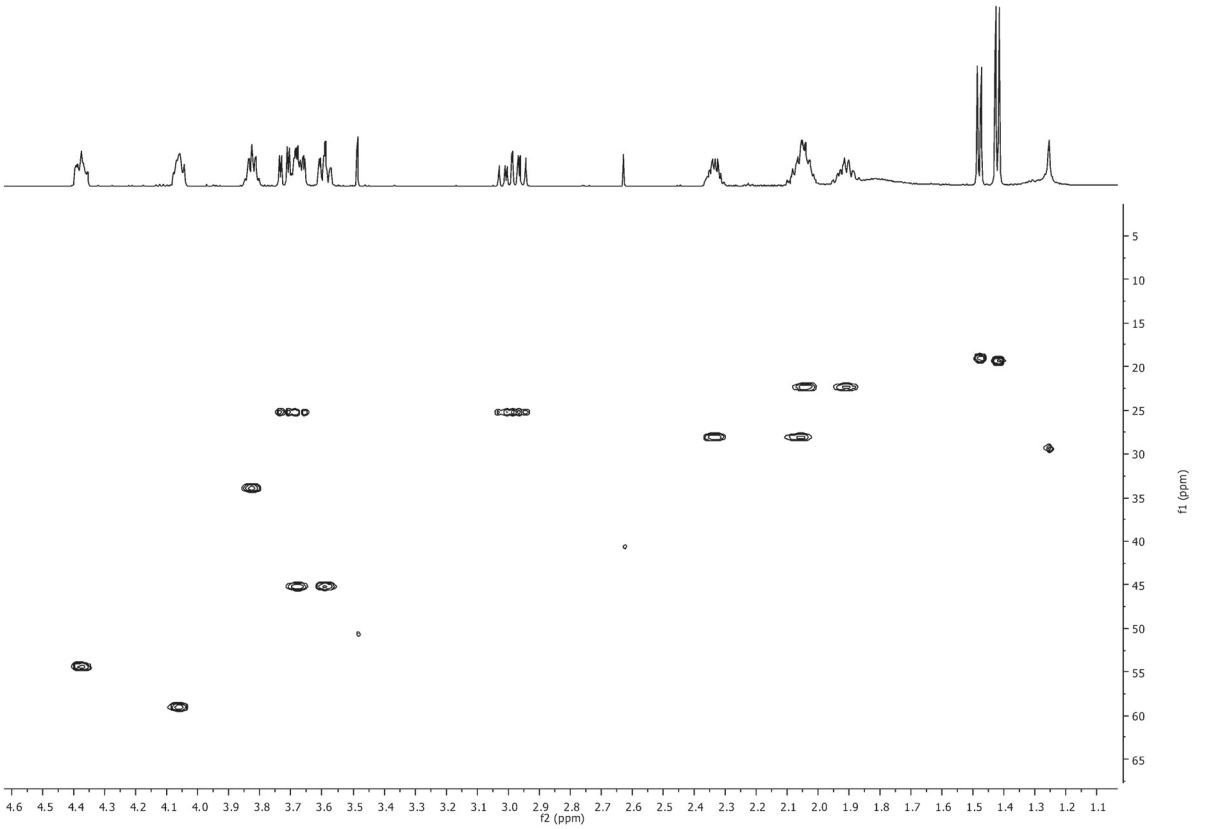


Figure S62. HSQC spectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

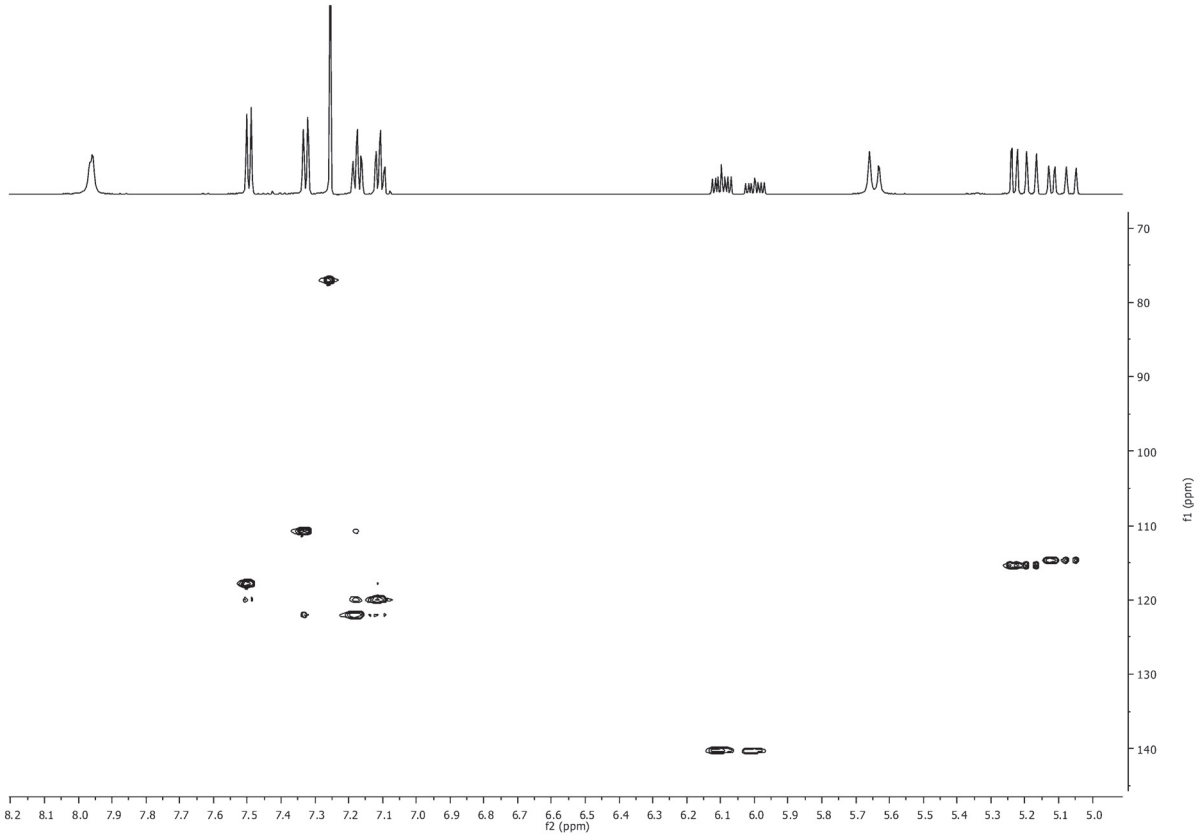


Figure S63. HSQC spectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

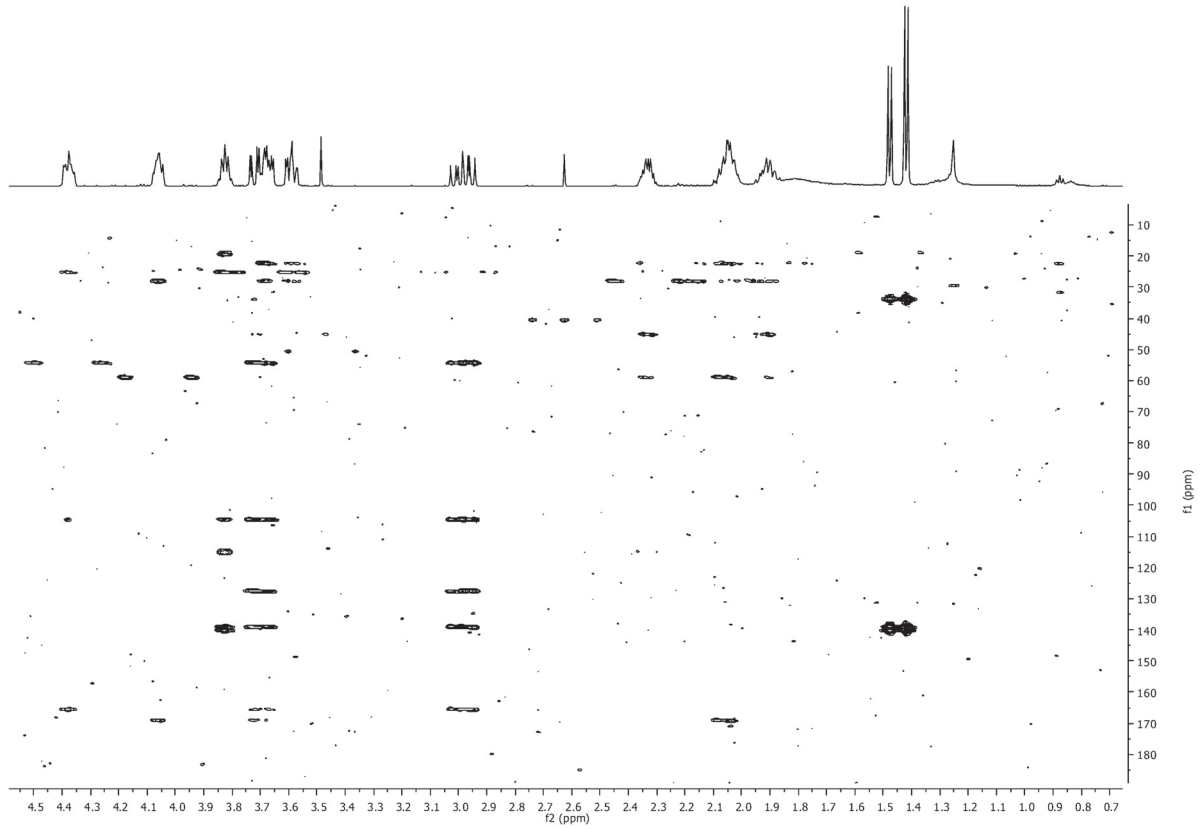


Figure S64. HMBC spectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

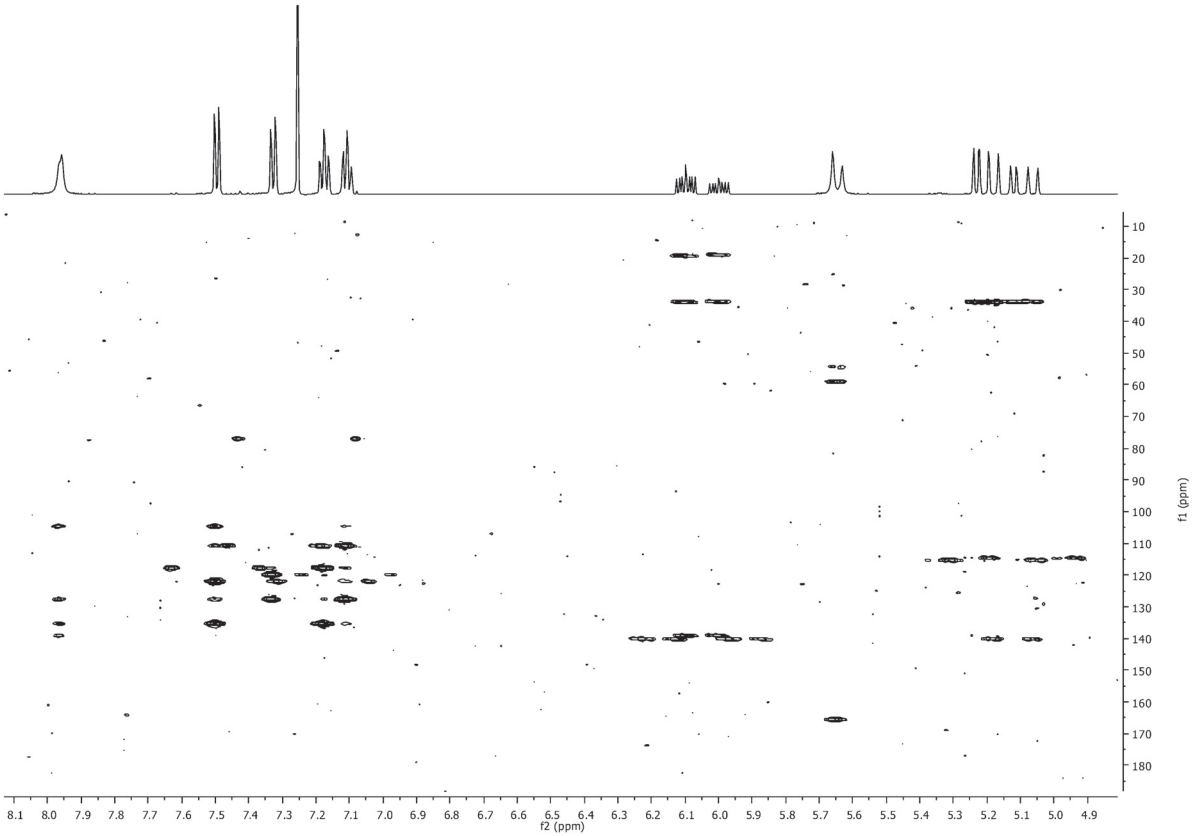


Figure S65. HMBCspectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

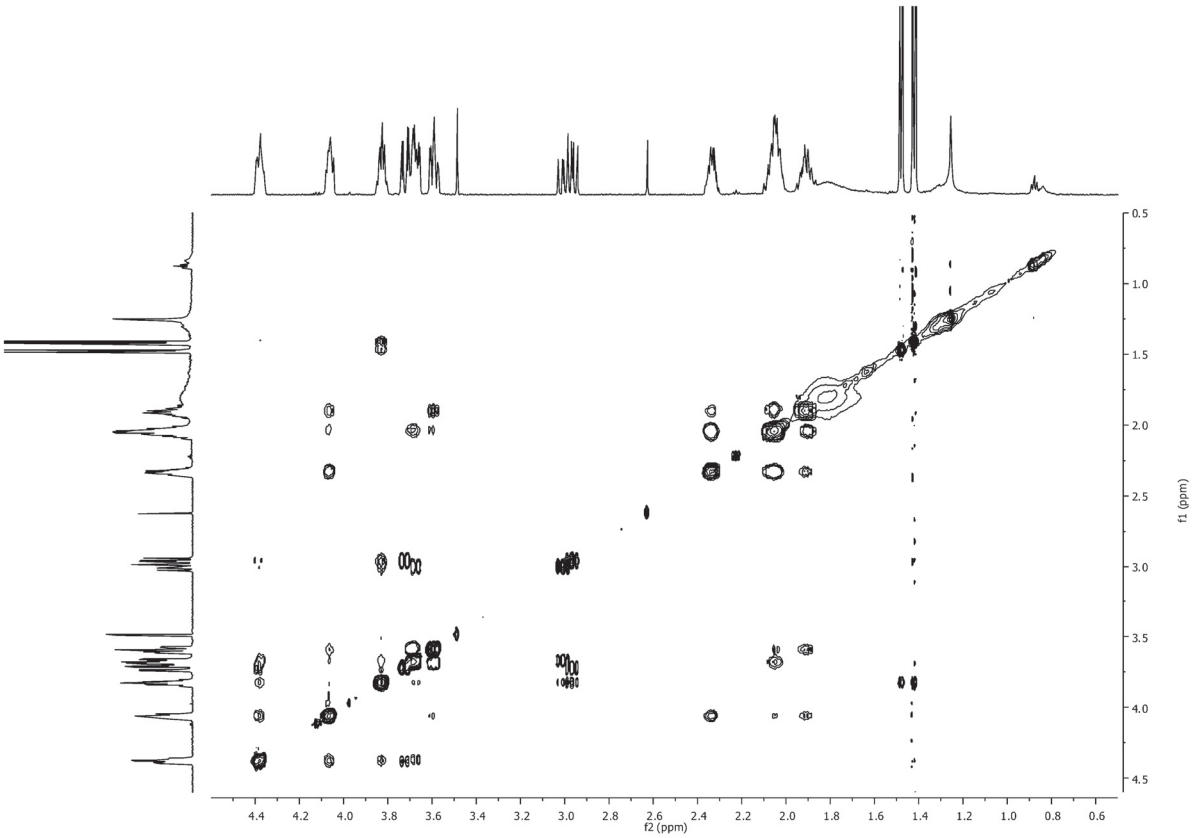


Figure S66. NOESY spectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

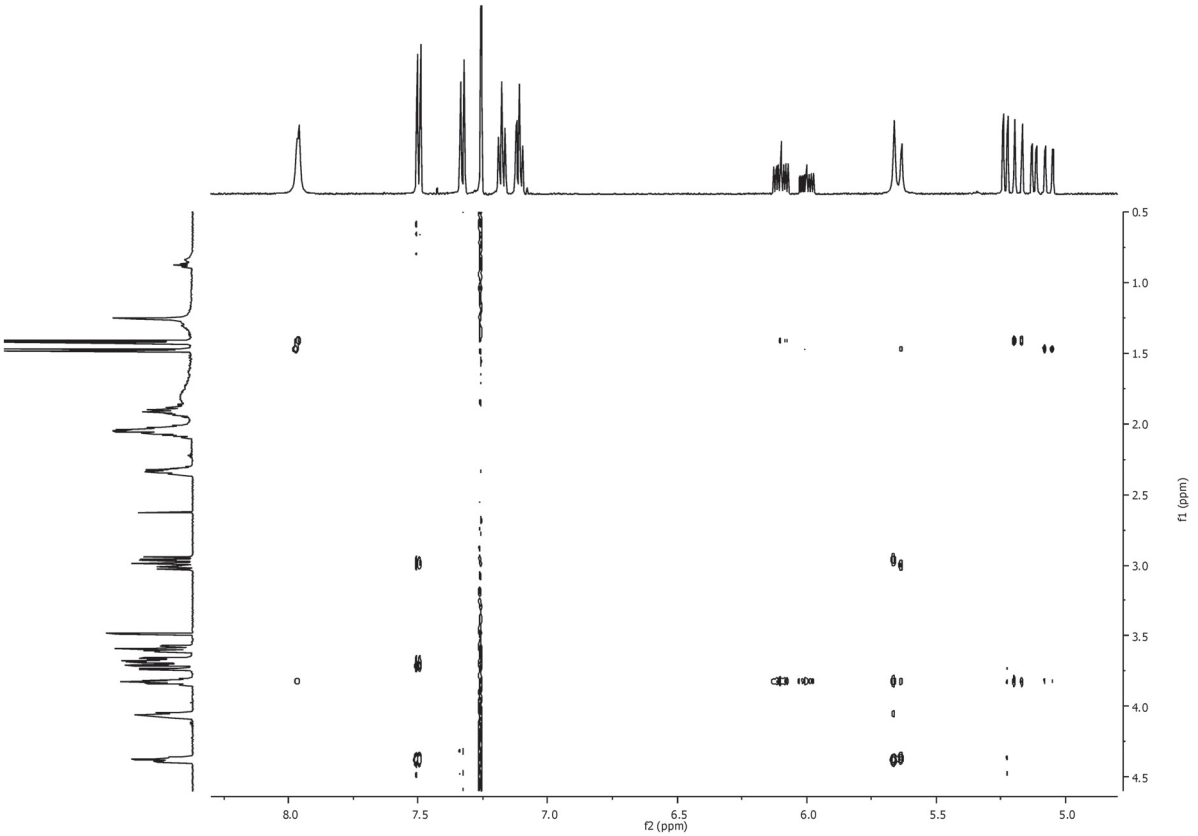


Figure S67. NOESY spectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

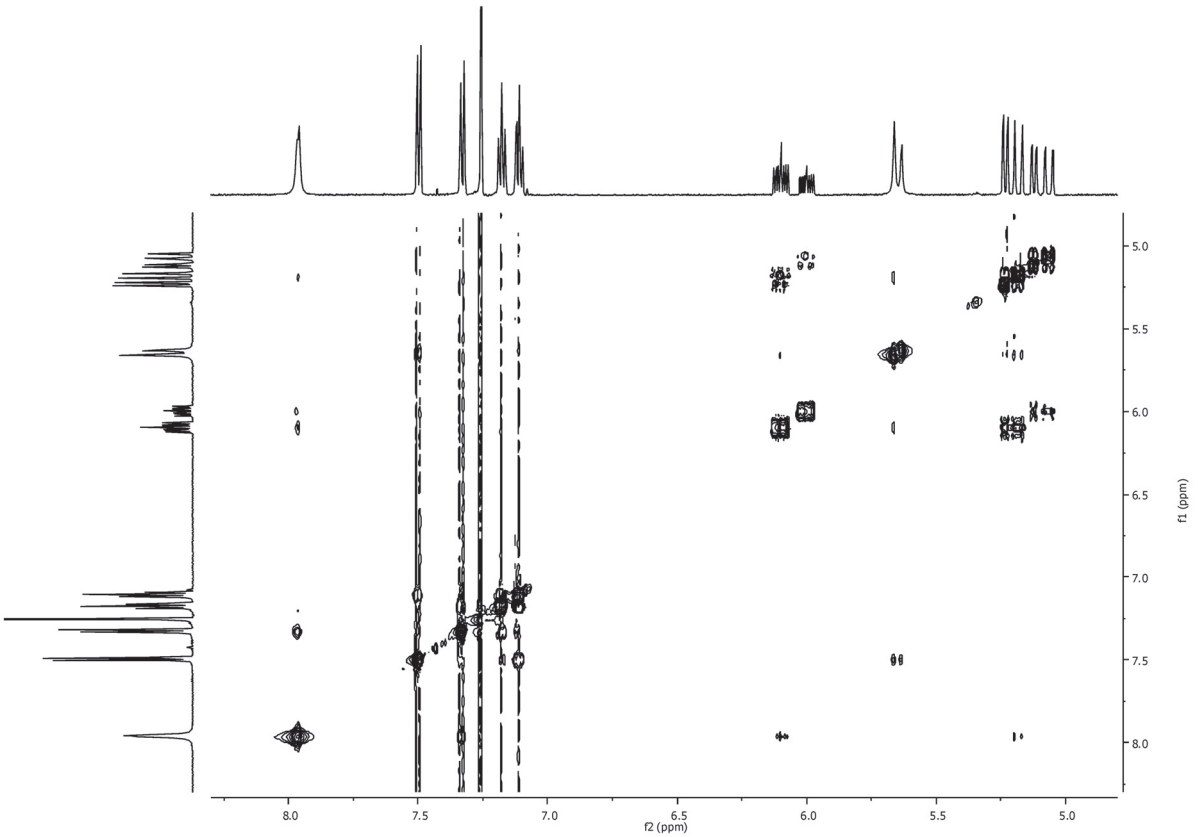


Figure S68. NOESY spectrum of *cyclo*-C2-(3'*R*-butenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1a+1b**) in $CDCl_3$.

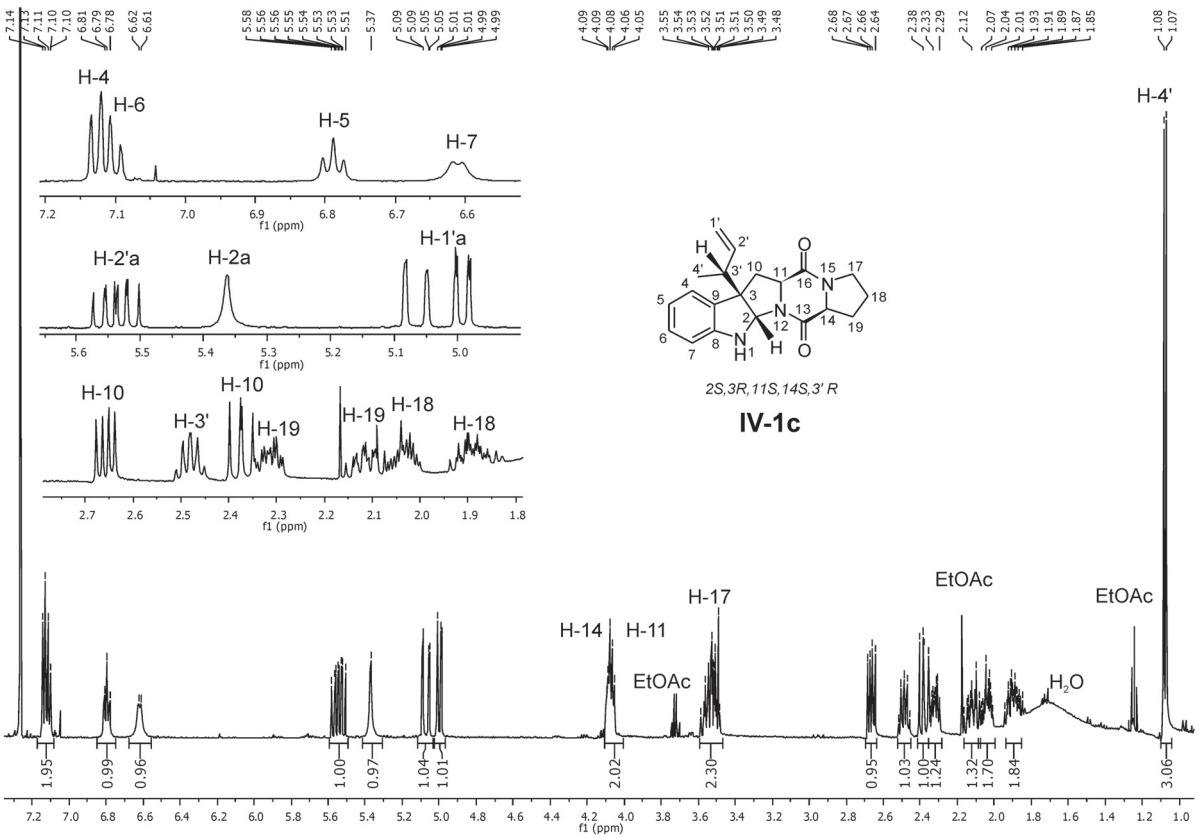


Figure S69. ^{1}H NMR spectrum of mixture *cyclo*-C3β-(3'*R*-butenyl-)-L-Trp-L-Pro (**IV-1c**) in $CDCl_3$.

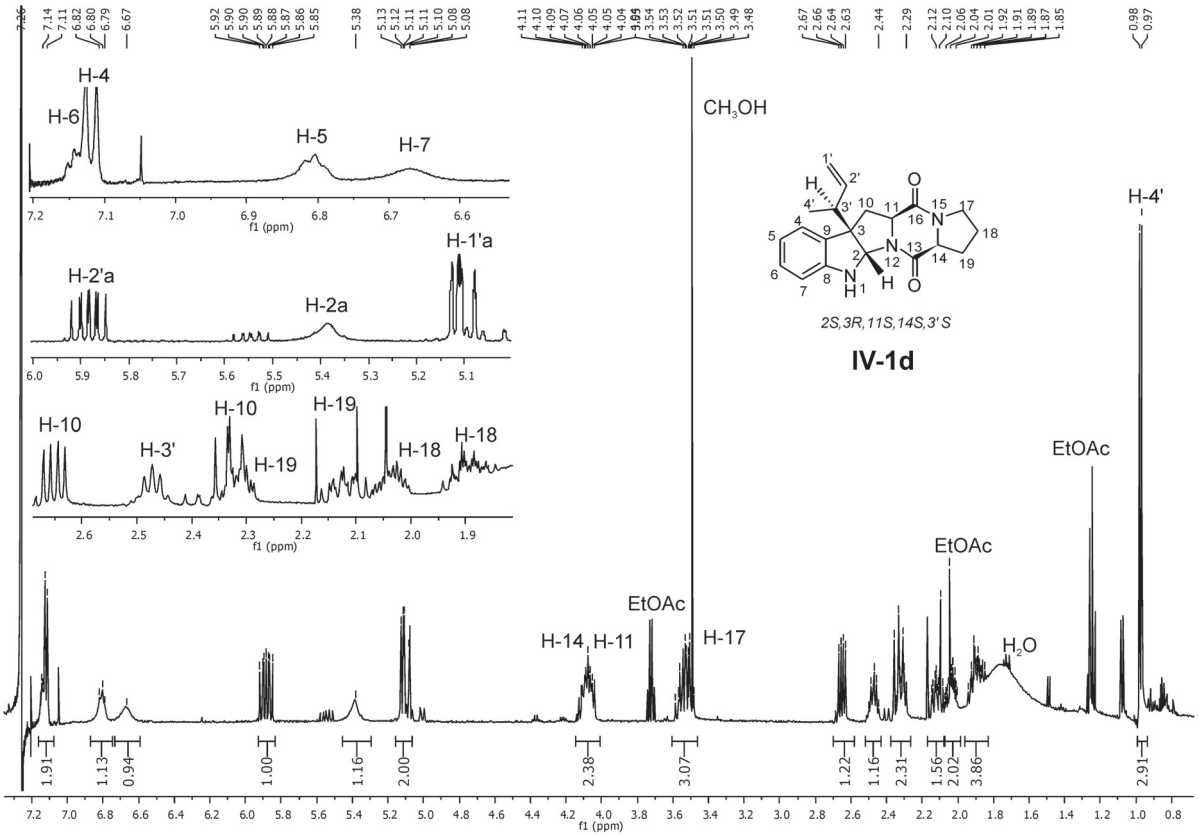


Figure S70. ^{1}H NMR spectrum of mixture *cyclo*-C3β-(3'*S*-butenyl-)-L-Trp-L-Pro (**IV-1d**) in $CDCl_3$.

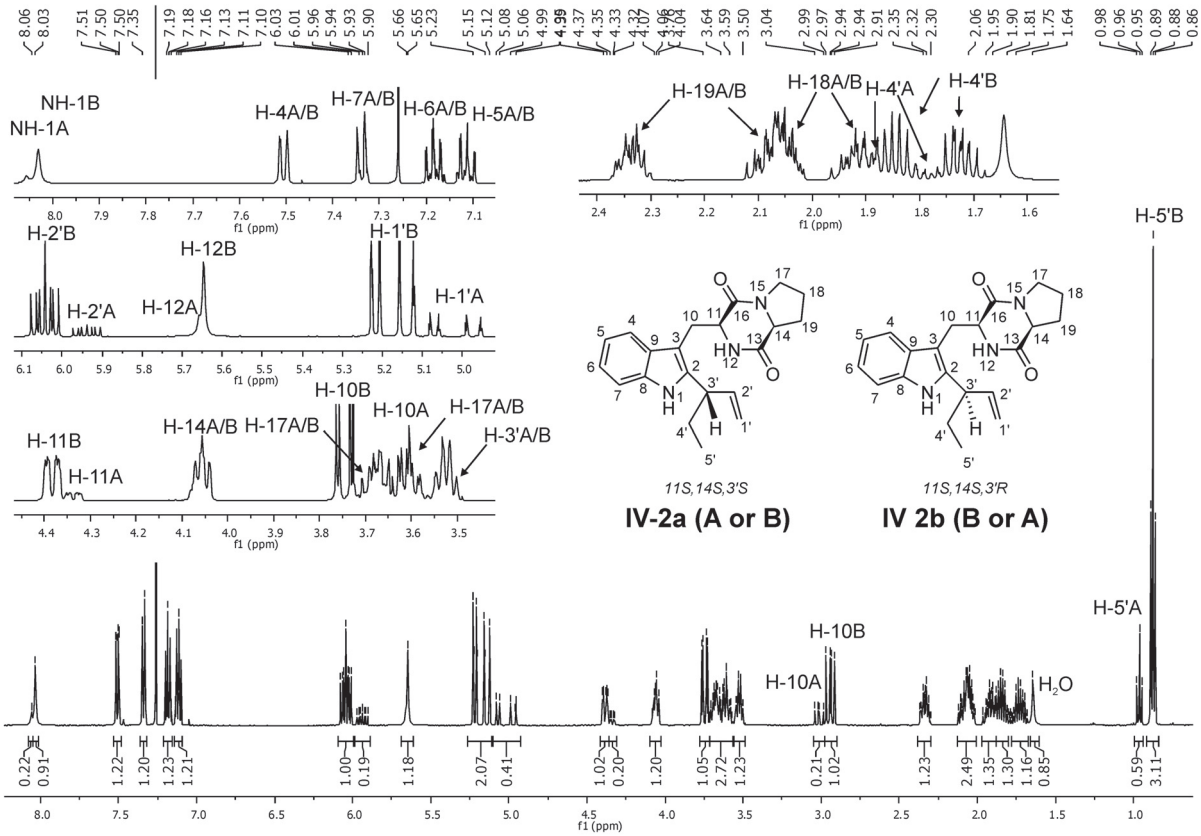


Figure S71. ^{1}H NMR spectrum of mixture *cyclo*-C2-(3'*R*-pentenyl-)-L-Trp-L-Pro and *cyclo*-C2-(3'*S*-pentenyl-)-L-Trp-L-Pro (**IV-2a+2b**) in $CDCl_3$.

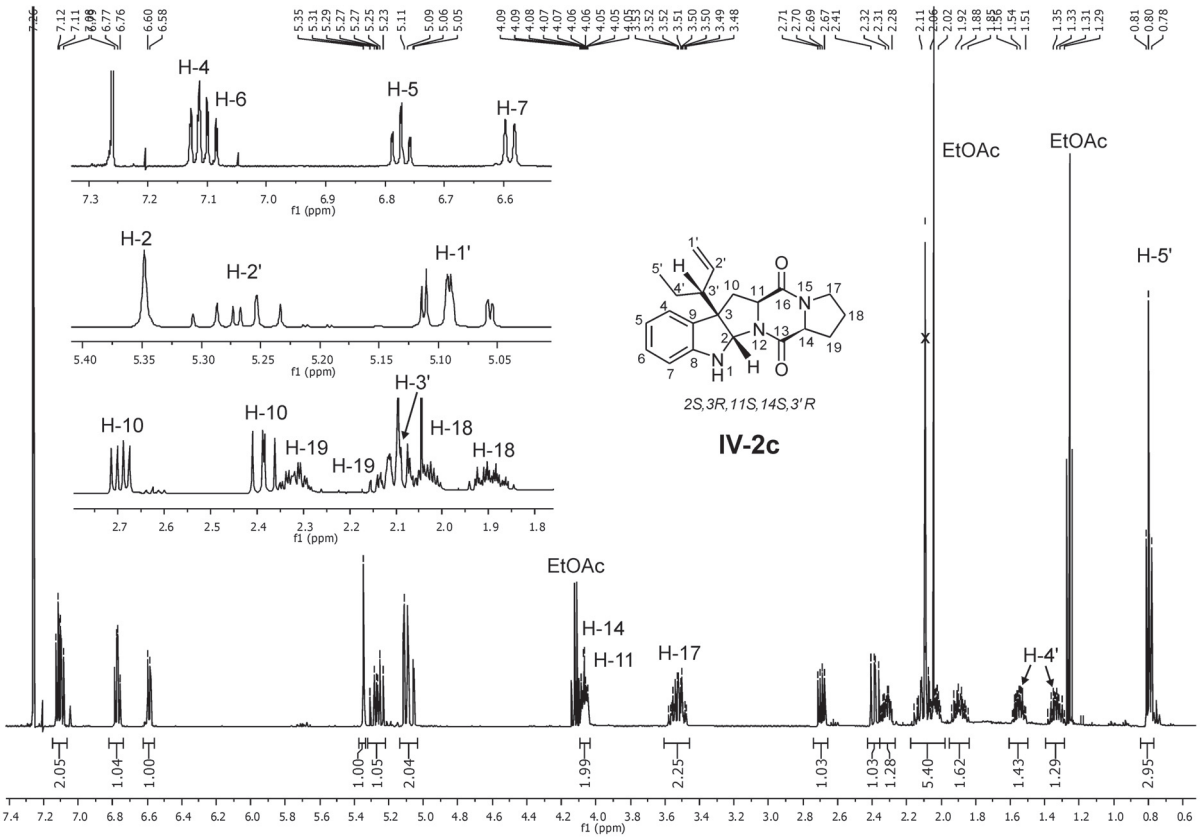


Figure S72. ^{1}H NMR spectrum of mixture *cyclo*-C3β-(3'*R*-pentenyl-)-L-Trp-L-Pro (**IV-2c**) in $CDCl_3$.

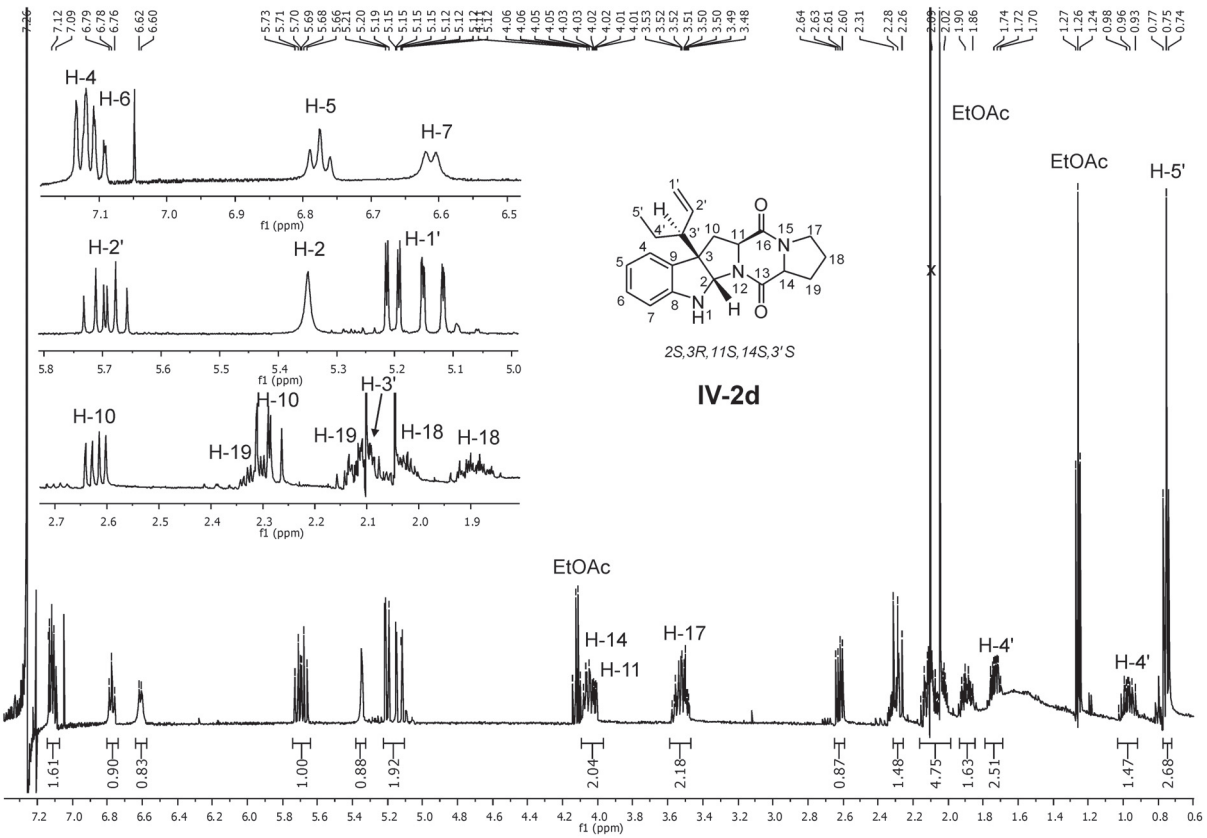


Figure S73. ^{1}H NMR spectrum of mixture *cyclo*-C3β-(3'*S*-pentenyl-)-L-Trp-L-Pro (**IV-2d**) in $CDCl_3$.

4.3. Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase

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Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase

Mike Liebhold and Shu-Ming Li*

Institut für Pharmazeutische Biologie und Biotechnologie, Philipps Universität Marburg Deutschhausstrasse 17a, 35037 Marburg, Germany

shuming.li@staff.uni-marburg.de

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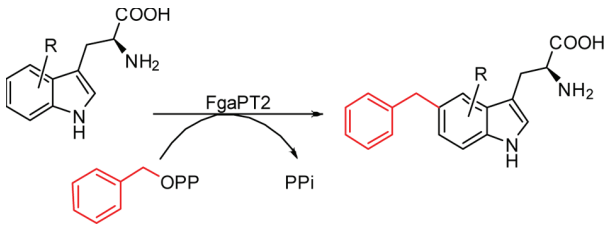
ABSTRACT



A big challenge in organic synthesis is to reach a high regioselectivity. Enzymes catalyze usually highly regiospecific reactions and can function as ideal biocatalysts for such purposes. Some secondary metabolite enzymes can even use distinctly different unnatural substrates and expand therefore their potential usage in chemoenzymatic synthesis. We report here the acceptance of benzyl diphosphate as an alkyl donor by the fungal dimethylallyl transferase FgaPT2 and the regiospecific enzymatic benzylation of tryptophan and several analogues.

Prenylated indole alkaloids such as notoamides, roquefortines, fumitremorgins, and ergot alkaloids are a large group of natural products with diverse chemical structures and strong biological activities.[1] These compounds are usually derived from L-tryptophan or derivatives thereof as backbones and prenyl moieties as modifications.[1d,e] The attachment of prenyl moieties ($n \times$ C5 units) usually increases the lipophilicity of the resulted products and strengthens their interaction with proteins and biomembranes. Prenyl moieties are often critical for the intriguing biological and pharmacological activities of the prenylated compounds.[2] Prenyl transfer reactions, i.e. prenylations, are catalyzed in nature by prenyltransferases, which can be divided into several subgroups.[3] One such subgroup comprises the members of the **dim**ethyl**a**llyl **t**ryptophan **s**ynthase (DMATS) superfamily. They are involved in the biosynthesis of secondary metabolites and mainly catalyze the transfer reactions of dimethylallyl moieties from dimethylallyl diphosphate (DMAPP) onto various positions of the indole ring of diverse indole derivatives including tryptophan, tryptophan-containing cyclic dipeptides, or complex structures.[4] For example, FgaPT2 is involved in the biosynthesis of ergot alkaloids, e.g. fumigaclavines in *Aspergillus fumigatus*, and catalyzes the prenylation of L-tryptophan at position C-4 in the presence of DMAPP (Scheme 1, a).[5] The prenylations catalyzed by the members of the DMATS superfamily are often Friedel–Crafts alkylations. Enzymes, which catalyze C-prenylations for all of the six positions (C-2 to C-7) of the indole ring, have been characterized in recent years.[4d] One common feature of these enzymes is their high flexibility toward their

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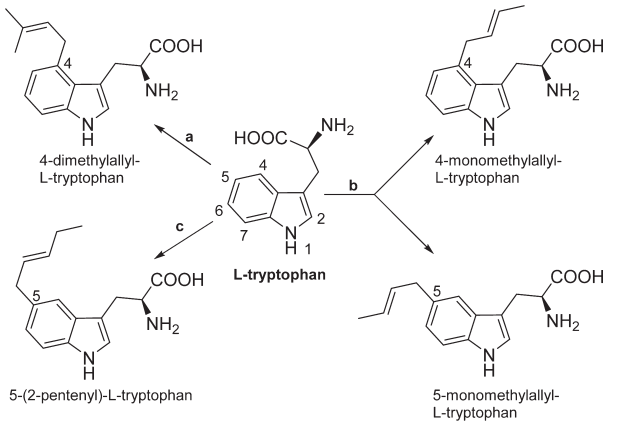
aromatic substrates. They accept not only structures similar to their natural substrates, usually L-tryptophan or tryptophan-containing cyclic dipeptides, but also significantly different structures such as flavonoids or hydroxynaphthalenes as prenyl acceptors.[4d,6] On the other hand, they display a strict substrate specificity toward their prenyl donor and usually only accept DMAPP as a substrate. One enzyme of this superfamily accepts geranyl diphosphate (GPP), but not DMAPP, as a substrate.[7] Acceptance of both DMAPP and GPP has been reported for AnaPT recently.[8] The low flexibility toward alkyl donors strongly prohibited their potential usage for chemoenzymatic synthesis and synthetic biology. In recent years, research on S-adenosyl-L-methionine (SAM) dependent methyl transferases has shown that an array of SAM analogues, with alkenyl, alkinyl, or aryl residues instead of a methyl group, were accepted as unnatural cofactors and alkylation agents.[9] We were intrigued to prove the ability of indole prenyltransferases for using unnatural alkyl donors. Therefore, we modified the DMAPP structure and tested the acceptance of several DMAPP analogues by enzymes of the DMATS superfamily.[10]

Our results with monomethylallyl diphosphate (MAPP, Scheme 1, b) and 2-pentenyl diphosphate (2-pen-PP, Scheme 1, c) showed that these unnatural alkyl donors were well accepted by a number of prenyltransferases of the DMATS superfamily, resulting in the formation of different alkylated derivatives (Scheme 1).[10] These works indicated that the limited flexibility of such enzymes toward their alkyl donors can be overridden in parts and therefore encouraged us to test alkyl donors with a significantly different structure to that of DMAPP. For this purpose, we successfully synthesized benzyl diphosphate from benzyl chloride according to a method described for GPP,[11] with a total conversion of 58% and a purity of over 90%, and used it as an alkyl donor for enzyme assays of nine indole prenyltransferases with their respective natural or reported best accepted aromatic substrates. The substrates used included L-tryptophan (**1a**) for the three L-tryptophan prenyltransferases FgaPT2, 5-DMATS, and 7-DMATS and cyclic dipeptides for six cyclic dipeptide prenyltransferases.[4d] The tested dipetides were (*R*)-benzodiazepindione for AnaPT, (*S*)-benzodiazepindione for CdpNPT, *cyclo*-L-Trp-L-Pro for FtmPT1 and BrePT, *cyclo*-L-Trp-L-Leu for CdpC3PT, and *cyclo*-L-Trp-L-Trp for CTrpPT.[4d]

Scheme 1. Alkylations of L-Tryptophan Catalyzed by FgaPT2



HPLC analysis revealed that benzyl diphosphate was much better accepted by FgaPT2, with a conversion of 58.1% after incubation with 10 μg of enzyme in a 100 μL assay at 37 °C for 16 h, than other enzymes. Product formation was detected for 5-DMATS, 7-DMATS, AnaPT, and CdpNPT with conversion yields between 1% and 3% (data not shown). No product formation was observed for other enzymes under the tested conditions (data not shown). Based on the initial success with **1a**, we tested the acceptance of 25 additional tryptophan derivatives (**2a**–**26a**) with modifications on the side chain or at the indole ring by FgaPT2 in the presence of benzyl diphosphate (Table S1). HPLC analysis showed that at least 16 of these compounds were accepted by FgaPT2 with conversion yields from 0.8% for 5-methyl-DL-tryptophan (**17a**) to 54.4% for L-abrine (**2a**). D-tryptophan (**13a**) was much poorer accepted than its L-form **1a**, with a relative conversion yield of 2.6%. Racemic tryptophan was consumed to a similar conversion yield as pure L- and D-tryptophan together, indicating that D-tryptophan was consumed to a similar degree as in the enzyme assay with pure D-isomer. In the incubation mixtures with other racemic substrates, the conversion yield of each of the enantiomers could not be determined in this study. Therefore, the sum of their conversion yields were estimated from the total amount of substrates. Conversion yields of $>10\%$ were observed for **1a** and eight derivatives (**2a**–**9a**), which were thereby chosen for isolation and structure elucidation (Figure S1 and Tables 1 and S1). Total conversions of 35.5% for α-methyl-DL-tryptophan (**7a**) to 82.5% for **1a** have been even achieved in the enzyme assays for product isolation with 40 μg of protein per 100 μL assay.

HPLC chromatograms of **1a**–**9a** clearly showed one product peak each (Figure S1). **2a**, 4-methyl-DL-tryptophan (**3a**), and L-β-homotryptophan (**4a**) were consumed to a comparable degree as **1a**. Interestingly, among the five tested C5-substituted tryptophan derivatives, only 5-hydroxy-L-tryptophan (**9a**) was well accepted by FgaPT2 in the presence of benzyl diphosphate. In contrast,

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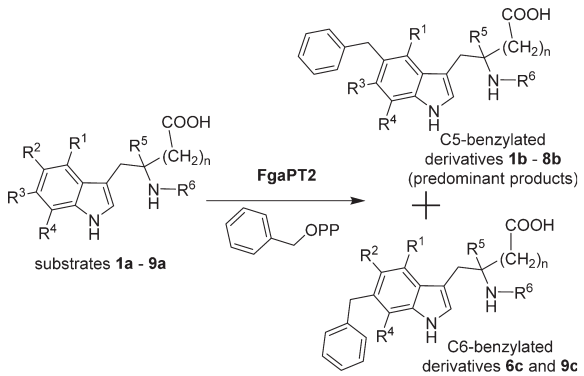
derivatives with substitutions at other positions of the indole ring, as in the cases of 4- (**3a**), 6- (**8a**), and 7-methyl-DL-tryptophan (**6a**) or 6-fluoro-DL-tryptophan (**5a**), were very well consumed. These observations could indicate C-5 of the indole ring as the favorable benzylation position by FgaPT2 in the presence of benzyl diphosphate. This hypothesis would also explain the high conversion of **3a** to **3b**. Interestingly, **5a** was much better accepted than **8a**, which could not be explained by different electron densities on the benzene ring. It could be speculated that the smaller fluorine atom would better fit in the reaction chamber than a methyl group or there are interactions between fluorine and amino acid residues in the reaction chamber.

For confirmation of the benzylation and benzylation position, the enzymatic products of the nine substrates (**1a**–**9a**) were isolated on HPLC and subjected to high resolution MS and NMR analyses. HR-EI-MS revealed that these compounds are 90 Da larger than their respective substrates and confirmed the presence of a benzyl residue in the structures of the enzymatic products. Detailed analysis of NMR spectra of the obtained enzymatic products (see Supporting Information for structure elucidation) confirmed the C5-benzylation in **1b**–**8b** from **1a**–**8a** (Table 1). From the incubation mixture of **6a**, an additional product **6c** with a C6-benzyl moiety was also identified. It seems that the methyl group at C7 of the indole ring reduced the regioselectivity of the FgaPT2 reaction. For 5-hydroxy-DL-tryptophan (**9a**), only the C6-benzylated derivative **9c** was isolated, indicating a complete shift in regioselectivity (Table 1). This proved that FgaPT2 catalyzed in most cases the transfer reaction of a benzyl moiety from benzyl diphosphate to C-5 of tryptophan and derivatives. If this position is blocked, the benzylation then took place at C-6. With its natural substrate DMAPP as the prenyl donor, shifting of the alkylation position was also observed, when the original prenylation position C-4 was blocked, as in the case of **3a**.[12]

As aforementioned, the prenyltransferase FgaPT2 catalyzes in nature the prenylation of L-tryptophan at position C-4.[5b] It has been demonstrated that, in the presence of its natural prenyl donor DMAPP, the overproduced and purified FgaPT2 accepted a large number of simple indole derivatives and tryptophan-containing cyclic dipeptides and catalyzed the regiospecific prenylation at C-4 of the indole rings.[13] In the presence of two unnatural DMAPP analogues MAPP and 2-pen-PP, FgaPT2 also catalyzed the alkylation of L-tryptophan. In the case of the larger alkyl donor 2-pen-PP, the alkylation position was shifted from C-4 to C-5 (Scheme 1). For the smaller MAPP, both C4- and C5-alkylations were observed (Scheme 1). In this study, we reported the acceptance of benzyl diphosphate by FgaPT2 and the highly regiospecific alkylation of tryptophan derivatives. As observed for 2-pen-PP,[10a] the alkylation position was shifted from C-4 to C-5. It seems that C-5 can be reached easier by the benzyl residue than C-4. Substances with substitution at this position were very poor substrates for FgaPT2 in the presence of benzyl diphosphate. One exception was 5-hydroxy-L-tryptophan, which was converted to a C6-benzylated derivative.

Table 1. Selected Substrates and Their Benzylated Products[a]



	yield	R^1	R^2	R^3	R^4	R^5	R^6	n	C5-benzylated	C6-benzylated
1a (L)	100.0	H	H	H	H	H	H	0	**1b**	–
2a (L)	93.6	H	H	H	H	H	**CH_3**	0	**2b**	–
3a (DL)	88.0	**CH_3**	H	H	H	H	H	0	**3b**	–
4a (L)	85.9	H	H	H	H	H	H	1	**4b**	–
5a (DL)	61.6	H	H	**F**	H	H	H	0	**5b**	–
6a (DL)	35.3	H	H	H	**CH_3**	H	H	0	**6b**	**6c**
7a (DL)	33.9	H	H	H	H	**CH_3**	H	0	**7b**	–
8a (DL)	21.3	H	H	**CH_3**	H	H	H	0	**8b**	–
9a (L)	31.2	H	**OH**	H	H	H	H	0	–	**9c**

[a] Relative yields are given in %; A total conversion yield of 58% was dermined for **1a** and defined as 100%. 100 μL assays contained 1 mM aromatic substrate, 2 mM benzyl diphosphate, 10 mM $CaCl_2$, 10 μg of FgaPT2, and 50 mM Tris-HCl (pH 7.5) and were incubated at 37 °C for 16 h. Configurations of the substrates are given in parentheses. – = Not detected.

Our results reported in this study provide experimental evidence that some prenyltransferases, at least FgaPT2 and several other enzymes including 5-DMATS, 7-DMATS, AnaPT, and CdpNPT, can also utilize alkyl donors with strongly modified structures from its natural substrate DMAPP for Friedel–Crafts alkylation. To the best of our knowledge, neither an acceptance of benzyl diphosphate by a prenyltransferase as an alkyl donor nor a one-step regioselective benzylation of tryptophan and derivatives by secondary metabolite enzymes has been reported previously.

Based on the results obtained from crystal structures of three indole prenyltransferases and site-directed mutagenesis experiments,[14] it was proposed that the prenyl transfer reactions catalyzed by prenyltransferases in the presence of DMAPP begin with the removal of pyrophosphate from enzyme-bound DMAPP and the formation of a positively charged dimethylallyl ion. The involvement of a cation during the prenylation was confirmed by a positional

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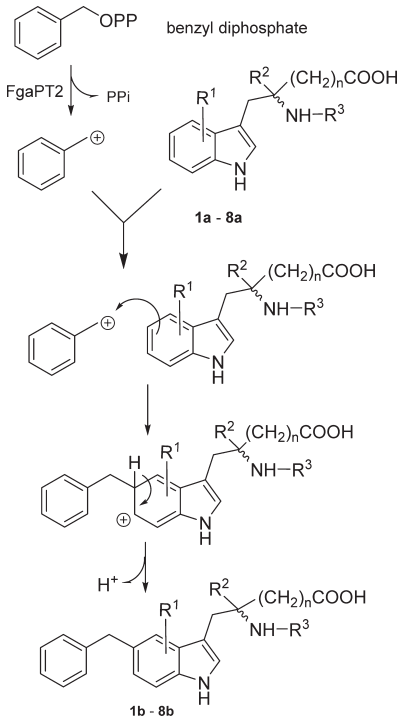
Scheme 2. Proposed Reaction Mechanism for Benzylation of L-Tryptophan and Derivatives (see Table 1 for detailed structures)



isotope exchange in isotopically labeled DMAPP.[15] The electron-rich aromatic ring would attack the C-1 or C-3 of this cation, resulting in the formation of different intermediates, which undergo various fates to enzyme-specific products.

In analogy to prenylations, a reaction mechanism can be postulated for the benzylation of tryptophan and derivatives by FgaPT2 in the presence of benzyl diphosphate (Scheme 2). It is plausible that the pyrophosphate of benzyl diphosphate is first removed by interaction with several basic amino acids.[14a] The resulted benzyl cation will be stabilized by interaction with tyrosine residues in the active center and by its mesomeric system. It seems that benzyl diphosphate and tryptophan derivatives are placed in positions relative to each other so that an attack of the indole residue with its C-5 becomes much easier than with its C-4 as in the case of DMAPP. Removal of a proton from C-5 rearomatizes the indole ring and results in the formation of C5-benzylated products. In the case of 5-hydroxy-L-tryptophan (**9a**), the C5 is blocked and C-6 is strongly activated by the hydroxyl group so that the attack took place from C-6 of the indole ring.

Comparison of the biochemical properties of the three dimethylallyltryptophan synthases FgaPT2, 5-DMATS, and 7-DMATS revealed different behaviors toward their substrates. 7-DMATS showed a much higher flexibility than FgaPT2 and 5-DMATS toward aromatic substrates. This enzyme accepted indole derivatives, hydroxynaphthalenes, and flavonoids as substrates.[6,16] On the other hand, the two DMAPP analogues MAPP and 2-pen-PP were very well accepted by FgaPT2 and 5-DMATS, but hardly by 7-DMATS.[10a] In this study, we showed that FgaPT2 can utilize benzyl diphosphate much better than 5- and 7-DMATS. These distinctive features increase their importance as biocatalysts in chemoenzymatic synthesis and expand their potential usage for Friedel–Crafts alkylations.

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Supporting Information Available. Experimental procedures, HPLC chromatograms, kinetic parameters, HR-EI-MS and NMR data. This material is available free of charge via Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase

Supporting Information

Mike Liebhold and Shu-Ming Li*

*Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17a, 35037 Marburg, Germany.

Experimental Section

Chemicals

Synthesis of benzyl diphosphate was carried out as described previously.[1] L-tryptophan (**1a**) was purchased from Roth (Karlsruhe, Germany); L-abrine (**2a**), 7-methyl-DL-tryptophan (**6a**), 6-methyl-DL-tryptophan (**8a**), L-tryptophan hydroxamate (**10a**), indole-3-propionic acid (**11a**), indole-3-lactic acid (**12a**), D-tryptophan (**13a**), 1-methyl-DL-tryptophan (**14a**), *N*-acetyl-DL-tryptophan (**16a**), 5-fluoro-L-tryptophan (**19a**), 5-bromo-DL-tryptophan (**20a**), indole-3-butyric acid (**21a**) and indole-3-acetic acid (**22a**) were purchased from Sigma Aldrich (Taufkirchen, Germany); L-β-homotryptophan (**4a**), 5-hydroxy-L-tryptophan (**9a**), 5-methoxy-DL-tryptophan (**18a**) and tryptamine (**26a**) were purchased from Fluka (Sigma Aldrich); *trans*-indole-3-acrylic acid (**15a**) and serotonin (**25a**) were purchased from Alfa Aesar (Karlsruhe, Germany); 4-methyl-DL-tryptophan (**3a**), α-methyl-DL-tryptophan (**7a**) and *N*-acetyl-L-tryptophan ethylester (**24a**) were purchased from Bachem (Bubendorf, Switzerland); 6-fluoro-DL-tryptophan (**5a**) and indole-3-pyruvic acid (**23a**) were purchased from Acros (Geel, Belgium); 5-methyl-DL-tryptophan (**17a**) was purchased from TCI (Eschborn, Germany).

Overproduction and purification of the recombinant protein His_8-FgaPT2

The recombinant protein His_8-FgaPT2 was prepared by cultivation of *Escherichia coli* BL21 (DE3) pLysS (Invitrogen, Karlsruhe, Germany) cells harboring the expression plasmid pIU18.[2]

The cells were cultivated in 2.5 L Erlenmeyer flasks containing 1 L liquid terrific broth (TB) medium and were supplemented with kanamycin (50 μg ml^{-1}). The cultures were grown for 2-3 h at 37 °C and 220 rpm till an OD_{600} of 0.7 to 0.8 was reached. After addition of IPTG to

a final concentration of 0.5 mM, the cells were further incubated at 22 °C for 6 h. Protein preparation and purification were carried out as described previously.[2]

Assays for determination of the enzyme activities

The reaction mixtures (100 µl) for determination of the enzyme activities contained 1 mM L-tryptophan or derivatives, 10 mM $CaCl_2$, 2 mM benzyl diphosphate, 1.0-1.5% (v/v) glycerol, 2.5-5.0% (v/v) dimethyl sulfoxide (DMSO), 50 mM Tris-HCl (pH 7.5) and 10 µg of purified recombinant protein. The reaction mixtures were incubated at 37 °C for 16 h. For HPLC analysis on RP-18 column, the reactions were terminated with 100 µl MeOH. Protein was then removed by centrifugation at 17.000 × g for 15 min.

Enzyme assays for isolation and structure elucidation

Assays for isolation of the enzymatic products were carried out in large scales (10 ml) containing 1 mM L-tryptophan or derivatives, 2 mM benzyl diphosphate, 10 mM $CaCl_2$, 0.0%-1.5% (v/v) glycerol, 0.0-2.5% (v/v) DMSO, 50 mM Tris-HCl (pH 7.5) and 0.2 mg or 0.4 mg of purified recombinant protein per ml assay. After incubation for 16 h at 37 °C, the reaction mixtures were terminated with 10 ml MeOH and protein was removed by centrifugation at 4.750 × g for 15 min. The supernatant was then concentrated using a rotary evaporator. ^{1}H-NMR spectra were taken from the residues for determination of the product yields. After evaporation of the NMR solvent, the samples were dissolved in a mixture of DMSO/MeOH and subjected to preparative HPLC equipped with RP-18 columns. Conversion yields were calculated by measuring the decrease of peak areas of substrate after incubation and by use of the absorption coefficient calculated from the signal ratios in the NMR spectra of the substrate/product mixtures in the isolation assays.

HPLC analysis and isolation of the enzymatic products for structure elucidation

The enzyme products were routinely analyzed by HPLC on an Agilent series 1200 (Agilent Technologies Deutschland GmbH, Böblingen, Germany) by using a Multospher 120 RP-18 column (250 × 4 mm, 5 µm, CS-Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 ml min^{-1}. Water (solvent A) and methanol (solvent B) were used as solvents for analysis and isolation of the enzymatic products. A linear gradient of 40-100% (v/v) solvent B in A in 15 min was used for analysis of the enzyme assays. The column was then washed with 100% solvent B in A for 5 min and equilibrated with 40% solvent B in A for another 5 min. Detection was carried out on a photo diode array detector.

The enzyme products were isolated on a Multospher 120 RP-18 column (250 × 10 mm, 5 µm, CS-Chromatographie Service, Langerwehe, Germany). Gradients of 60-80% (v/v) in 15 min (**9c**), 70-90% in 20 min (**5b**) and 70-90% (v/v) in 15 min (all other compounds) solvent B in A at 2.5 ml min^{-1} were used. Each run comprised an isocratic section solvent B in A prior to the gradient for 5 min. After each run, the column was washed with 100% solvent B in A for 5 min followed by 5 min of equilibration with the corresponding condition at the beginning of the next run.

NMR and MS analyses as well as structure elucidation

NMR including two-dimensional COSY and NOESY spectra were recorded on a JEOL ECA-500 500 MHz spectrometer (JEOL Germany GmbH, Munich, Germany). Chemical shifts were referred to the signals of DMSO-d6 at 2.50 ppm as well as of CD_3OD at 3.30 ppm. The spectra were processed with MestRENova 6.0.2. The isolated compounds were also analyzed by electrospray ionization mass spectrometry (ESI-MS) on a Q-Trap 2000 (Life Technologies Ltd, Paisley PA4 9RF, United Kingdom) and high resolution electron impact mass

spectrometry (HR-EI-MS) on an Auto SPEC (Waters MS Technology Centre, Manchester, United Kingdom).

In the ^{1}H-NMR spectrum of **1a** (in CD_3OD), the signals of the indole moiety at δ_H 7.59 (1H, d, 0.8, H-4), δ_H 7.26 (1H, d, 8.3, H-7), δ_H 7.16 (1H, s, H-2) and δ_H 6.96 (1H, dd, 8.3, 1.6, H-6) were very similar to those of 5-dimethylallyl-L-tryptophan (5-DMAT).[3] Solely the signal of H-4 was slightly (approx. 0.10 ppm) downfield shifted. The signals of H-10 and H-11 in the spectra of **1b** and 5-DMAT were also nearly identical. These data proved unequivocally the C5-benzylation of **1a** by FgaPT2 in the presence of benzyl diphosphate. The slight difference of the chemical shifts of H-4 in **1b** from that of 5-DMAT could be explained by the replacement of the dimethylallyl moiety by an electron-rich benzyl residue. Comparison of the ^{1}H-NMR data of **2b**, **4b** – **7b** with those of **1b** as well as of the corresponding C5-prenylated derivatives[3] revealed the same order and coupling pattern of the signals for aromatic protons and therefore verified the attachment of the benzyl residue to the indole ring at position C-5. Solely the signal of H-2 of **7b** was slightly downfield shifted into the signals of the benzyl moiety.

In the ^{1}H-NMR spectrum of **8b,** two of three singlets for one aromatic proton each were observed at 7.56 and 7.15 ppm, indicating a benzylation at C-4 or C-5. In the NOESY spectrum of **8b** (Figure S4) correlations between the singlet at δ_H 7.56 ppm (1H, s) and signals of both H-10 at 3.50 ppm and 3.09 ppm (moderate), H-11 at 3.86 ppm (moderate) as well as H-1' at 4.09 ppm (strong) were clearly observed. No correlation was detected between the singlet at δ_H 7.56 ppm (1H, s) and the signal of H-13 (methyl group) at 2.22 ppm. Strong correlations were observed between H-13 and H-1' as well as H-13 and the singlet at 7.15 ppm. These correlations can only be explained for a derivative with the benzyl residue at position C-5 (**8b**) with the singlet at 7.56 ppm for H-4 and the singlet at 7.15 ppm for H-7.

This explanation was also supported by the correlations of the H-2 signal at 7.10 ppm and those of H-10 and H-11.

One doublet in the spectrum of **3b** at 6.92 ppm with a coupling constant of 8.3 Hz indicates an alkylation at C-5 or C-7 of the indole ring. The chemical shifts and coupling pattern of the aromatic protons of the benzyl residue in **8b** and **3b** were very similar. They differed clearly from those of most of the other products reported in this study. This could be explained by the *ortho*-position of a methyl group to the benzyl moiety. Due to the hindrance of a free rotation of the benzyl moiety, the protons of H-2´and H-6´ as well as H-3´and H-5´ differ from each other. It is therefore plausible that the benzyl unit in **3b** is attached to C-5 of the indole ring.

In addition to the product **6b**, an additional product **6c** was detected in the reaction mixture of **6a**. One singlet at 7.15 ppm and two doublets at 6.86 and 6.67 ppm with a coupling constant of 7.2 Hz indicated a C4- or C6-benzylation. The chemical shifts and coupling pattern of the aromatic protons of its benzyl residue were very similar to those of **8b** and **3b,** indicating a probable benzylation at the *ortho*-position of the methyl group. We therefore assigned the benzylation to C-6 in **6c.**

In the ^{1}H-NMR spectrum of **9c,** three singlets for one aromatic proton each were observed at δ_H 7.04 (1H, s), δ_H 7.01 (1H, s) and δ_H 6.98 (1H, s), which proved the attachment of the benzyl moiety at C-6 of the indole ring, corresponding to prenylation position of the enzyme product of a 6-DMATS from *Streptomyces sp.* SN-693.[4]

Determination of the kinetic parameters

Assays for determination of the kinetic parameters of L-tryptophan contained 1 mM of benzyl diphosphate, 10 mM $CaCl_2$, 0.15% (v/v) glycerol, 50 mM Tris-HCl (pH 7.5) and L-tryptophan in final concentrations of 0.005 to 2 mM. Assays for determination of the kinetic parameters of benzyl diphosphate contained 1 mM of L-tryptophan, 10 mM $CaCl_2$, 0.15% (v/v) glycerol,

50 mM Tris-HCl (pH 7.5) and benzyl diphosphate in final concentrations of 0.01 to 2 mM. A protein amount of 3 µg, an incubation time of 45 min and an incubation temperature of 37 °C were used in both cases. The reactions were terminated with 100 µl MeOH. Protein was then removed by centrifugation at 17.000 x g for 15 min.

Michaelis-Menten kinetics were determined by Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots. K_M values of 12.6 µM and 95.8 µM were calculated for L-tryptophan and benzyl diphosphate, respectively. The turnover number k_{cat} was found to be 0.014 s^{-1}. In comparison to the value of 8 µM with DMAPP as alkyl donor, the K_M value of L-tryptophan was only slightly increased in the presence of benzyl diphosphate. In contrast, the affinity of FgaPT2 to its natural substrate DMAPP with a K_M of 4 µM was much higher than to benzyl diphosphate.[5] However, the affinity of FgaPT2 to benzyl diphosphate is still high enough to be used as a catalyst in the chemoenzymatic synthesis.

References

(1) Woodside, A. B.; Huang, Z.; Poulter, C. D. *Org. Synth.* **1988,** *66*, 211.

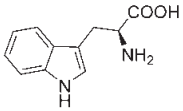
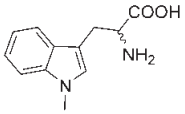
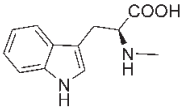
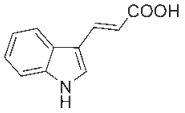
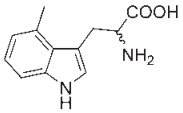
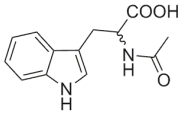
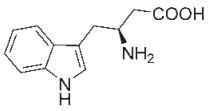
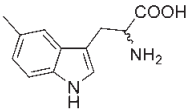
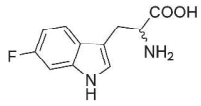
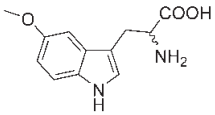
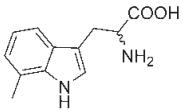
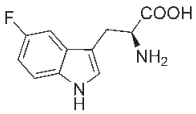
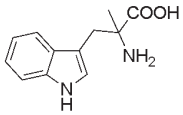
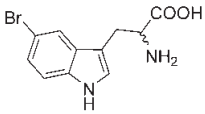
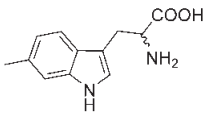
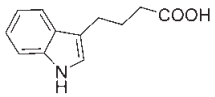
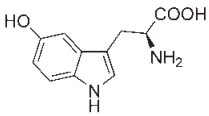
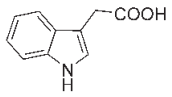
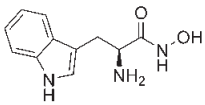
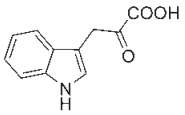
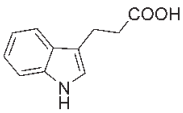
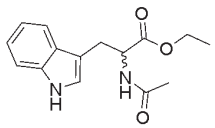
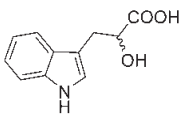
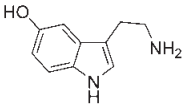
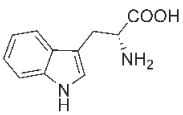
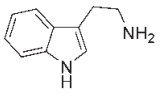
(2) Steffan, N.; Unsöld, I. A.; Li, S.-M. *Chembiochem* **2007,** *8*, 1298.

(3) Yu, X.; Liu, Y.; Xie, X.; Zheng, X.-D.; Li, S.-M. *J. Biol. Chem.* **2012,** *287*, 1371.

(4) Takahashi, S.; Takagi, H.; Toyoda, A.; Uramoto, M.; Nogawa, T.; Ueki, M.; Sakaki, Y.; Osada, H. *J. Bacteriol.* **2010,** *192*, 2839.

(5) Unsöld, I. A.; Li, S.-M. *Microbiology* **2005,** *151*, 1499.

Table S1. Enzyme activity of FgaPT2 towards L-tryptophan and derivatives.

substrates[a]	structures	rel. conv.[b] [%]	substrates[a]	structures	rel. conv.[b] [%]
L-tryptophan (**1a**)		100	1-methyl-DL-tryptophan (**14a**)		2.1
L-abrine (**2a**)		93.6	*trans*-indole-3-acrylic acid (**15a**)		2.1
4-methyl-DL-tryptophan (**3a**)		88.0	*N*-acetyl-DL-tryptophan (**16a**)		1.5
L-ß-homo-tryptophan (**4a**)		85.9	5-methyl-DL-tryptophan (**17a**)		1.4
6-fluoro-DL-tryptophan (**5a**)		61.6	5-methoxy-DL-tryptophan (**18a**)		n.d.
7-methyl-DL-tryptophan (**6a**)		35.3	5-fluoro-L-tryptophan (**19a**)		n.d.
α-methyl-DL-tryptophan (**7a**)		33.9	5-bromo-DL-tryptophan (**20a**)		n.d.
6-methyl-DL-tryptophan (**8a**)		31.2	indole-3-butyric acid (**21a**)		n.d.
5-hydroxy-L-tryptophan (**9a**)		21.3	indole-3-acetic acid (**22a**)		n.d.
L-tryptophan hydroxamate (**10a**)		14.5	indole-3-pyruvic acid (**23a**)		n.d.
indole-3-propionic acid (**11a**)		5.2	*N*-acetyl-DL-tryptophan ethylester (**24a**)		n.d.
indole-3-lactic acid (**12a**)		3.3	serotonin (**25a**)		n.d.
D-tryptophan (**13a**)		2.6	tryptamine (**26a**)		n.d.
DL-tryptophan (**1a+13a**)	see **1a**+**13a**	105.2			

[a] for measuring of the activity 10 µg enzymes were used per 100 µl assay; [b] the relative conversion of L-tryptophan refers to an absolute conversion of 58.0%; n.d.:not detected

Table S2. HR-EI-MS data of the enzyme products of FgaPT2 with benzyl diphosphate and L-tryptophan or derivatives.

			HR-EI-MS data		
compound	*benzylation position*	*chemical formula*	*calculated*	*measured*	*deviation[ppm]*
1b	C-5	$C_{18}H_{18}N_2O_2$	294.1368 [M^+]	294.1385 [M^+]	-5.8
2b	C-5	$C_{19}H_{20}N_2O_2$	308.1525 [M^+]	308.1540 [M^+]	-4.9
3b	C-5	$C_{19}H_{20}N_2O_2$	308.1525 [M^+]	308.1537 [M^+]	-3.9
4b	C-5	$C_{19}H_{20}N_2O_2$	308.1525 [M^+]	308.1524 [M^+]	0.3
5b	C-5	$C_{18}H_{17}N_2O_2F_1$	312.1274 [M^+]	312.1261 [M^+]	4.2
6b+c[a]	C-5 + C-6	$C_{19}H_{20}N_2O_2$	308.1525 [M^+]	308.1520 [M^+]	1.6
7b	C-5	$C_{19}H_{20}N_2O_2$	308.1525 [M^+]	308.1537 [M^+]	-3.9
8b	C-5	$C_{19}H_{20}N_2O_2$	308.1525 [M^+]	308.1531 [M^+]	-2.0
9c	C-6	$C_{18}H_{18}N_2O_3$	310.1317 [M^+]	310.1299 [M^+]	5.8

[a] MS data were obtained from the mixture of both compounds.

Table S3. ^{1}H-NMR data of benzyl diphosphate.

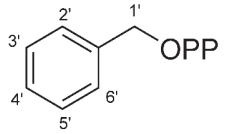
	benzyl diphosphate 
	$\delta_{H,}$ *multi,* J
H-1‘	5.08, d, 6,8
H-2‘	7,58, d, 7.1
H-3‘	7.53, t, 7.4
H-4‘	7.47, t, 7.2
H-5’	7.53, t, 7.4
H-6’	7,58, d, 7.1
solvent	4.79 (D_2O)

Table S4. ^{31}P-NMR data of benzyl diphosphate.

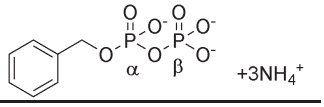
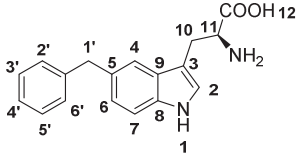
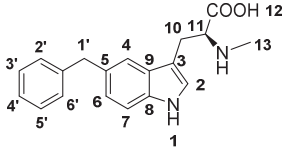
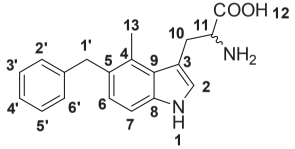
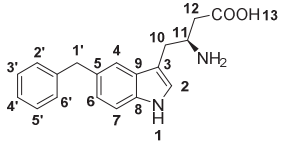
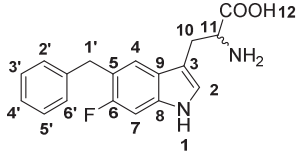
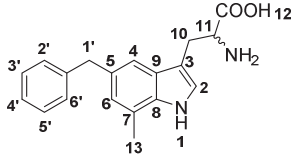
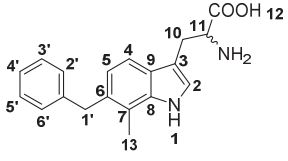
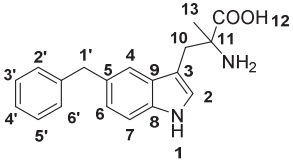
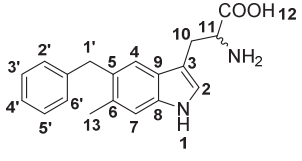
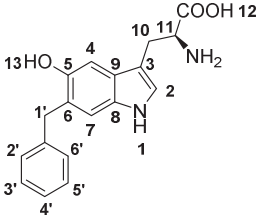
	benzyl diphosphate 
	$\delta_{P,}$ *multi,* J
P_α	-10.14, d, 21.2
P_β	-8.48, d, 21.2

Table S5. ^{1}H-NMR data of benzylated L-tryptophan (**1b**) and tryptophan derivatives **2b** to **5b.**

Comp	**5-benzyl-L-tryptophan (1b)** 	**5-benzyl-L-abrine (2b)** 	**5-benzyl-4-methyl-DL-tryptophan (3b)** 	**5-benzyl-L-β-homotryptophan (4b)** 	**5-benzyl-6-fluoro-DL-tryptophan (5b)** 
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
NH-1	-	10.78, s	-	10.75, s	10.83, s	-
2	7.16, s	7.17, d, 2.2	7.19, s	7.13, m	7.16, s	7.15, s
4	7.59, dd, 0.8	7.43, s	7.55, dd, 1.5, 0.6	-	7.45, s	7.60, d, 7.3
5	-	-	-	-	-	-
6	6.96, dd, 8.3, 1.6	6.92, dd, 8.3, 1.6	6.96, dd, 8.4, 1.5	6.92, d, 8.3	6.93, dd, 8.3, 1.5	-
7	7.26, d, 8.3	7.25, m	7.26, dd, 8.4, 0.6	7.13, m	7.26, d	7.04, d, 10.8
10	3.49, dd, 15.2, 4.0 3.08, dd, 15.2, 9.8	3.29, dd, 15.1, 3.9 2.88, dd, 15.1, 9.5	3.45, dd, 15.6, 4.7, 0.7 3.25, dd, 15.6, 8.0	3.61, dd, 15.8, 3.0 2.89, dd, 15.8, 10.7	2.22, dd, 16.2, 3.7 2.06, dd, 16.2, 9.7	3.46, dd, 15.1, 4.1 3.08, dd, 15.1, 9.6
11	3.84, dd, 9.8, 4.0	3.41, dd, 9.5, 3.9	3.80, dd, 8.0, 4.7	[a / b]	[a / b]	3.81, dd, 9.6, 4.1
12	-	-	-	-	2.91, dd, 14.4, 6.5 2.81, dd, 14.4, 7.7	-
13	-	-	2.55, s	2.53, s	-	-
1´	4.04, s	3.99, s	4.04, s	4.03, d, 1.8	3.96, s	4.05, s
2´	7.21, m	7.25, m	7.21, m	7.13, m	7.24, m	7.21, m
3´	7.21, m	7.25, m	7.21, m	7.24, dd, 8.1, 7.2	7.24, m	7.21, m
4´	7.11, m	7.14, m	7.12, m	7.13, m	7.14, m	7.11, m
5´	7.21, m	7.25, m	7.21, m	7.24, dd, 8.1, 7.2	7.24, m	7.21, m
6´	7.21, m	7.25, m	7.21, m	7.13, m	7.24, m	7.21, m
solvent	3.30 (CD_3OD)	2.50 (DMSO-d6)	3.30 (CD_3OD)	2.50 (DMSO-d6)	2.50 (DMSO-d6)	3.30 (CD_3OD)

[a] overlapping signals with that of water, [b] signal observed in COSY NMR

Table S6. ^{1}H NMR Data of benzylated tryptophan derivatives **6b** to **9c** and **6c**.

Comp	**5-benzyl-7-methyl-DL-tryptophan (6b)**	**6-benzyl-7-methyl-DL-tryptophan (6c)**	**5-benzyl-α-methyl-DL-tryptophan (7b)**	**5-benzyl-6-methyl-DL-tryptophan (8b)**	**6-benzyl-5-hydroxy-L-tryptophan (9c)**
					
Pos.	$\delta_{H,}$ *multi*, *J*	$\delta_{H,}$ *multi*, *J*	$\delta_{H,}$ *multi*, *J*	$\delta_{H,}$ *multi*, *J*	$\delta_{H,}$ *multi*, *J*
NH-1	-	-	-	-	-
2	7.16, s	7.15, s	7.21, m	7.10, m	7.04, s
4	7.43, d, 0.7	6.86, dd, 7.2, 0.7[c]	7.50, d, 0.7	7.56, s	6.98, s
5	-	6.67, d, 7.2[c]	-	-	-
6	6.77, s	-	6.96, dd, 8.3, 1.5	-	-
7	-	-	7.27, d, 8.3	7.15, s	7.01, s
10	3.49, ddd, 15.3, 3.9, 0.6	3.60, dd, 15. 3, 3.8	[a / b]	3.50, ddd, 15.2, 3.9, 0.7	3.43, ddd, 15.1, 3.8, 0.4
	3.08, dd, 15.3, 9.7	2.96, dd, 15.3, 10.4	3.21, d, 14.9	3.09, dd, 15.2, 9.8	3.03, dd, 15.1, 10.0
11	3.84, dd, 9.7, 3.9	[a]	-	3.86, dd, 9.8, 3.9	3.81, dd, 10.0, 3.8
13	2.41, s	2.46, s	1.51, s	2.22, s	
1′	4.01, s	4.48, d, 16.2	4.05, s	4.09, s	4.01, d, 6.7
		4.36, d, 16.2			
2′	7.21, m	7.12, m	7.21, m	7.10, m	7.20, m
3′	7.21, m	7.19, dd, 7.8, 7.0	7.21, m	7.19, dd, 8.1, 6.8	7.20, m
4′	7.11, m	7.11, m	7.12, m	7.10, m	7.11, m
5′	7.21, m	7.19, dd, 7.8, 7.0	7.21, m	7.19, dd, 8.1, 6.8	7.20, m
6′	7.21, m	7.12, m	7.21, m	7.10, m	7.20, m
solvent	3.30 (CD_3OD)	3.30 (CD_3OD)	3.30 (CD_3OD)	3.30 (CD_3OD)	3.30 (CD_3OD)

[a] overlapping signals with those of solvent, [b] signal observed in COSY NMR; [c] signals with the same letter are interchangeable.

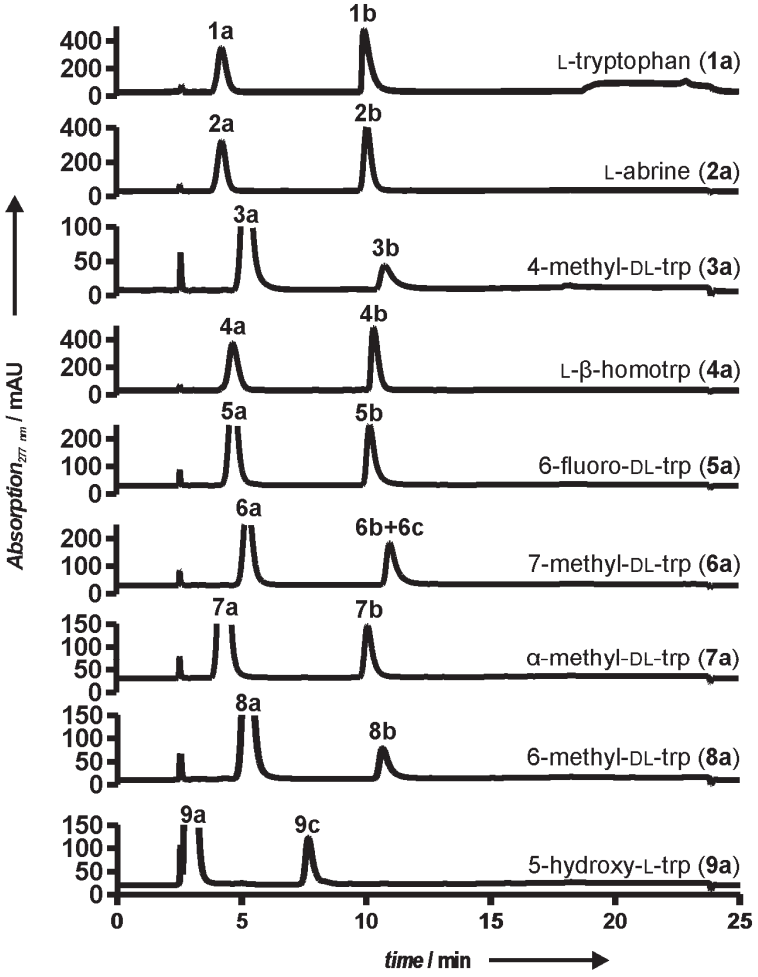


Figure S1. HPLC analysis of incubation mixtures of benzyl diphosphate with tryptophan and derivatives. The assays of 100 µl with 10 µg FgaPT2 were incubated at 37 °C for 16 h.

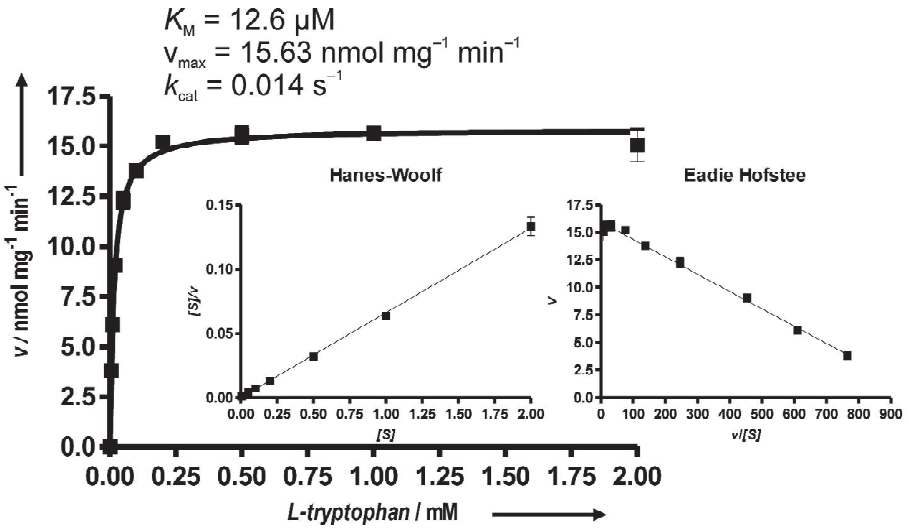


Figure S2. Dependence of the product formation of the FgaPT2 reaction on L-tryptophan in the presence of benzyl diphosphate.

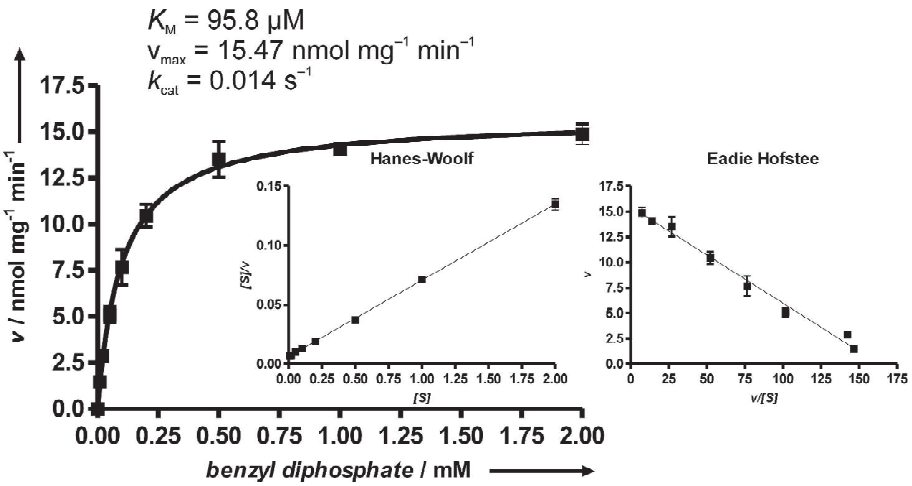


Figure S3. Dependence of the product formation of the FgaPT2 reaction on benzyl diphosphate in the presence of L-tryptophan.

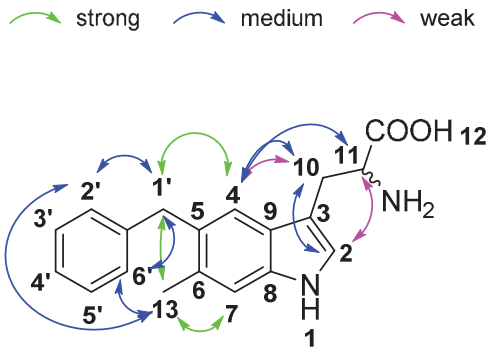


Figure S4. NOESY correlations of 5-benzyl-6-methyl-DL-tryptophan (**8b**).

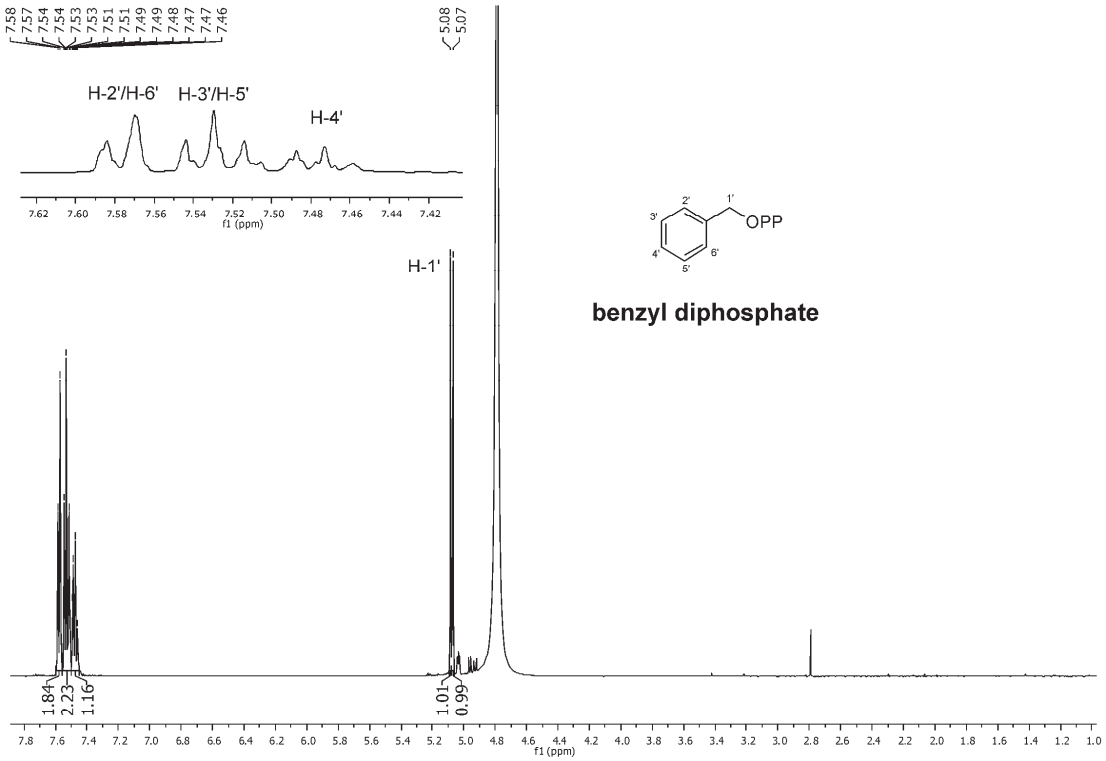


Figure S5. ^{1}H-NMR spectrum of benzyl diphosphate in D_2O.

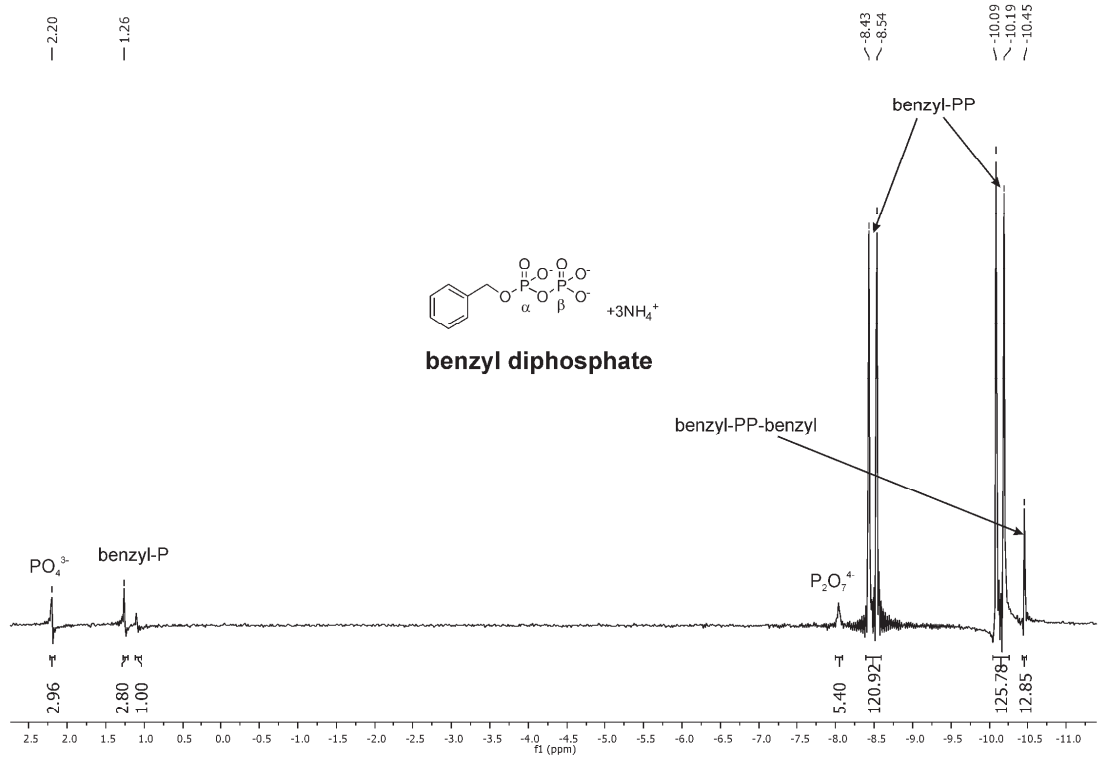


Figure S6. ^{31}P-NMR spectrum of benzyl diphosphate in D_2O.

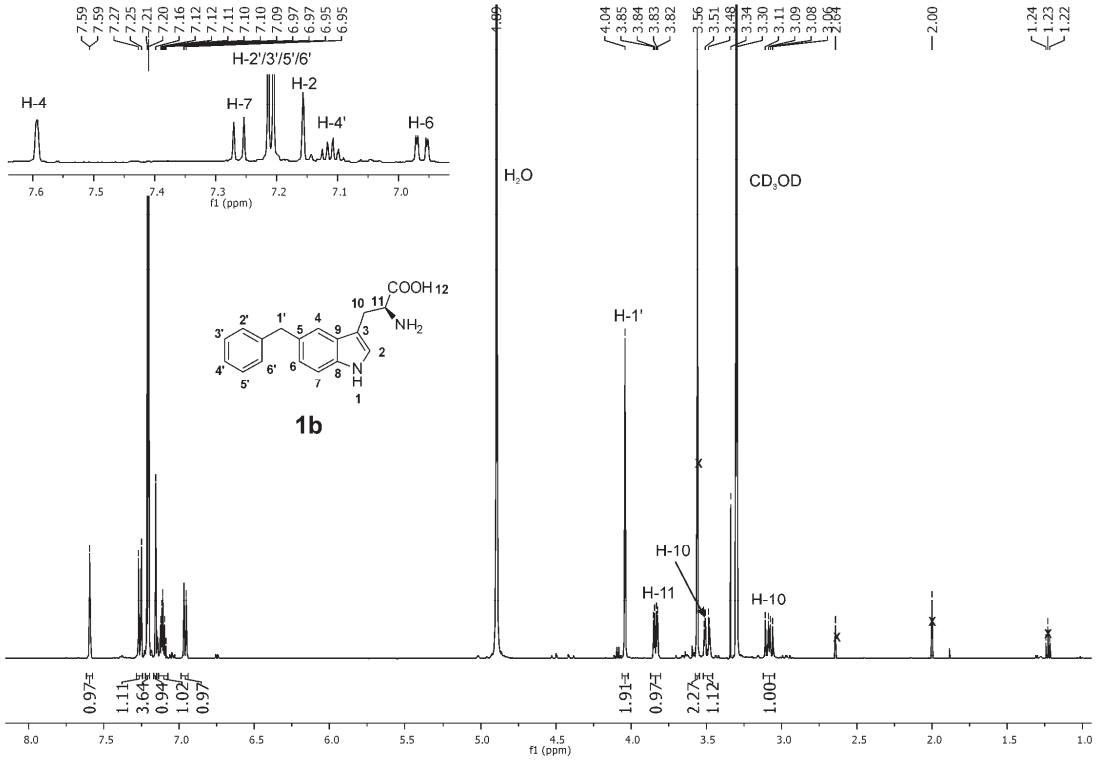


Figure S7. [1]H-NMR spectrum of 5-benzyl-L-tryptophan (**1b**) in CD_3OD.

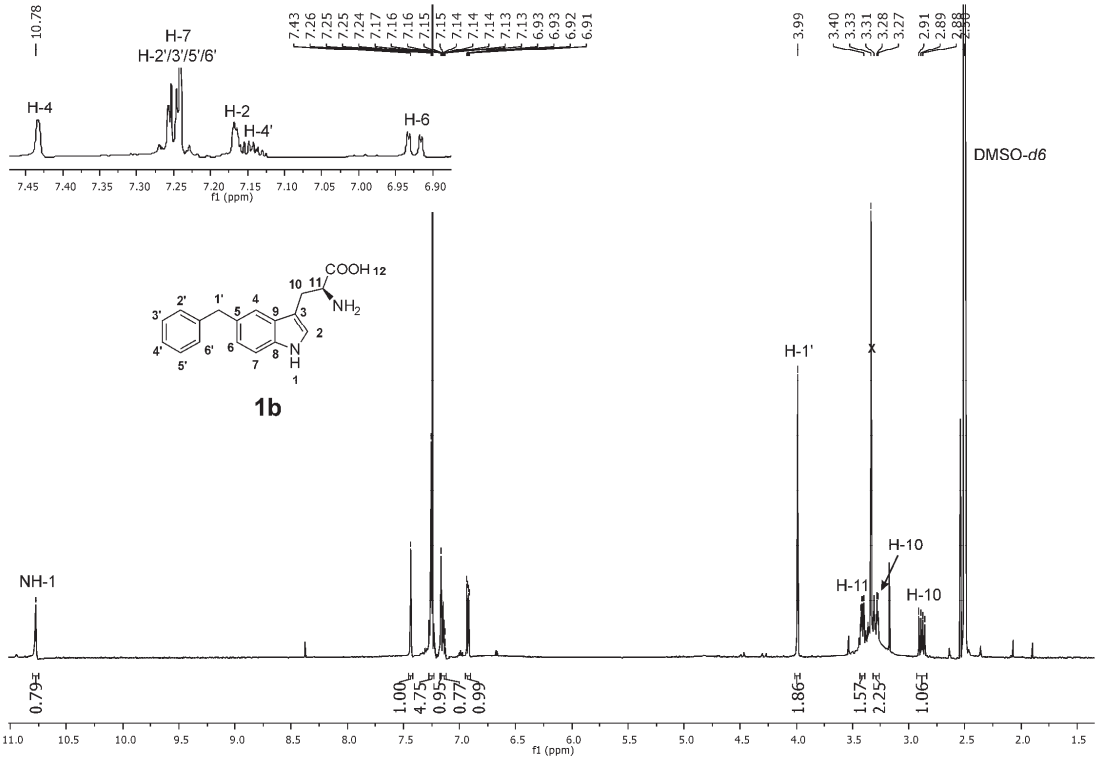


Figure S8. [1]H-NMR spectrum of 5-benzyl-L-tryptophan (**1b**) in DMSO-d6.

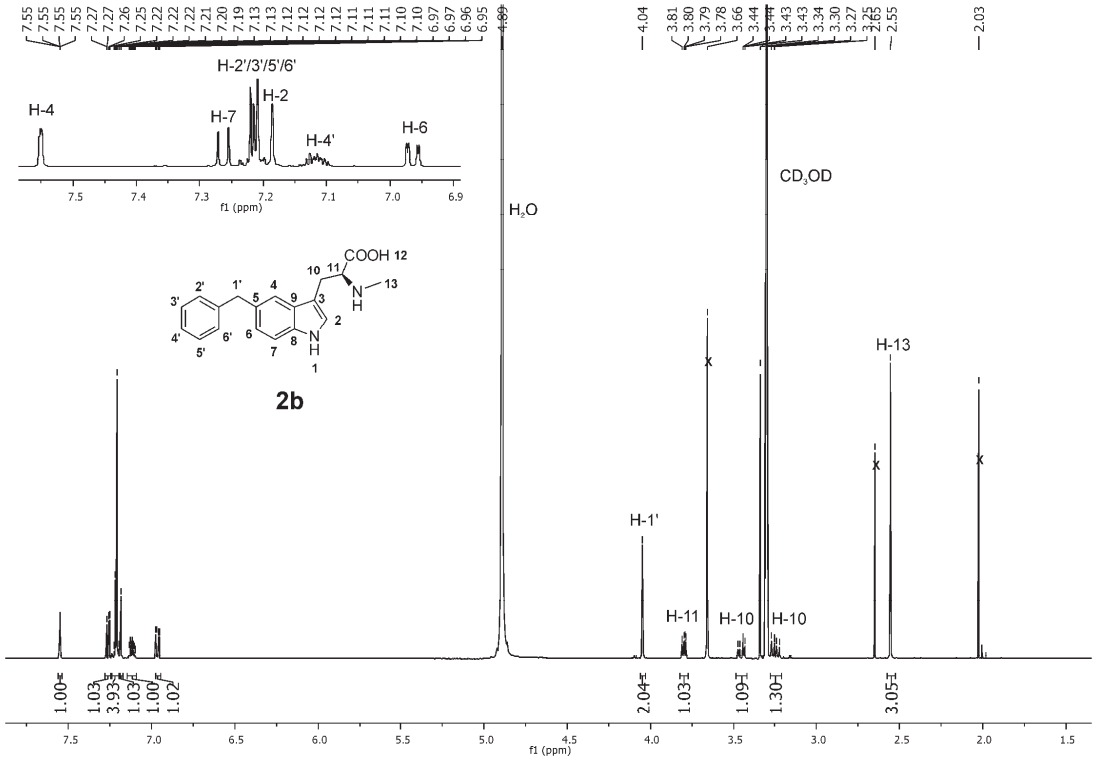


Figure S9. ^{1}H-NMR spectrum of 5-benzyl-L-abrine (**2b**) in CD_3OD.

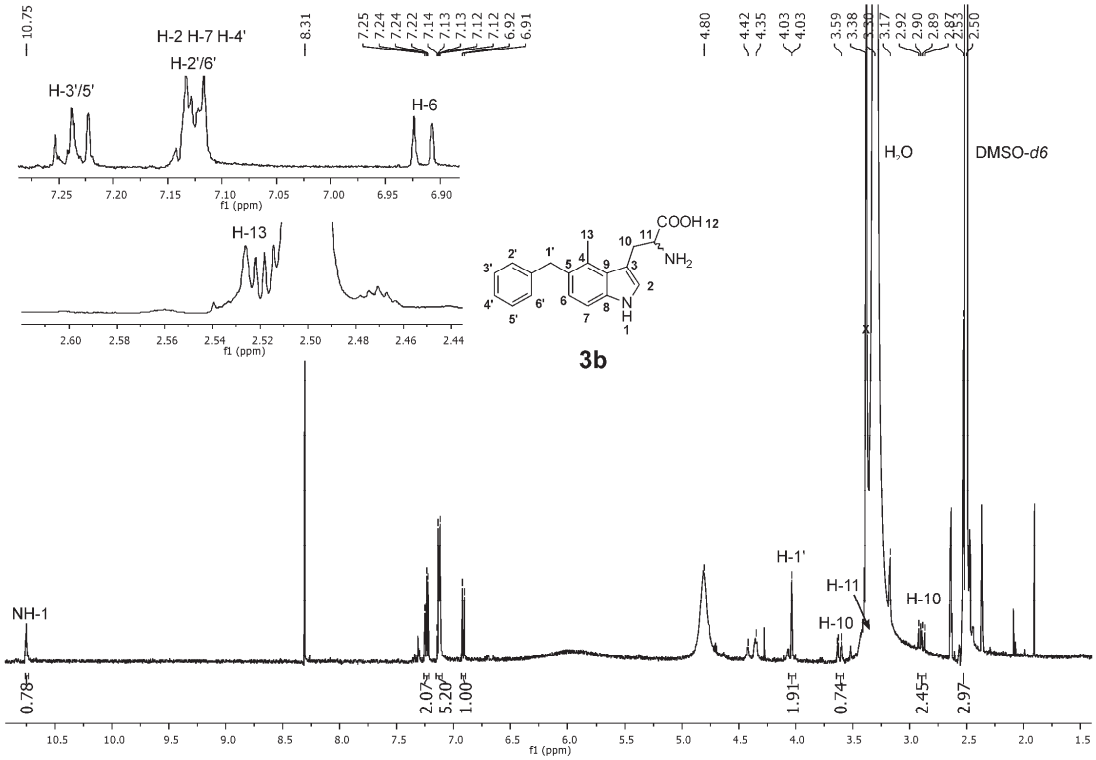


Figure S10. ^{1}H-NMR spectrum of 5-benzyl-4-methyl-DL-tryptophan (**3b**) in DMSO-d6.

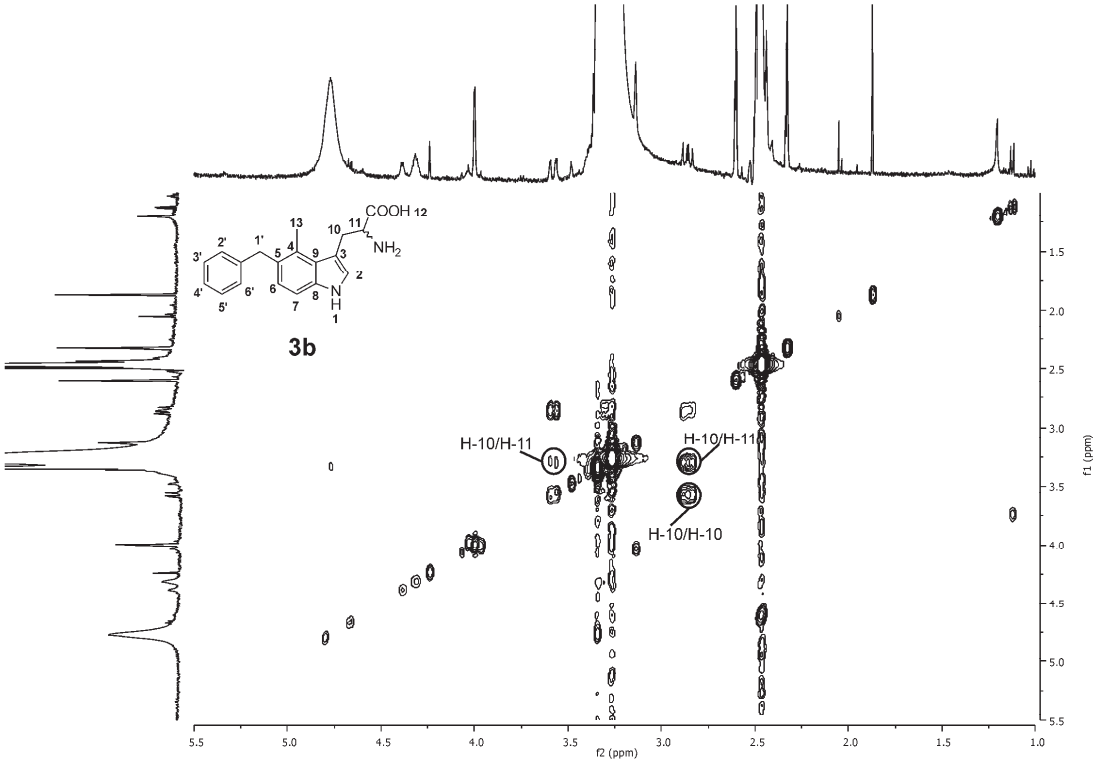


Figure S11. COSY spectrum of 5-benzyl-4-methyl-DL-tryptophan (**3b**) in DMSO-d6.

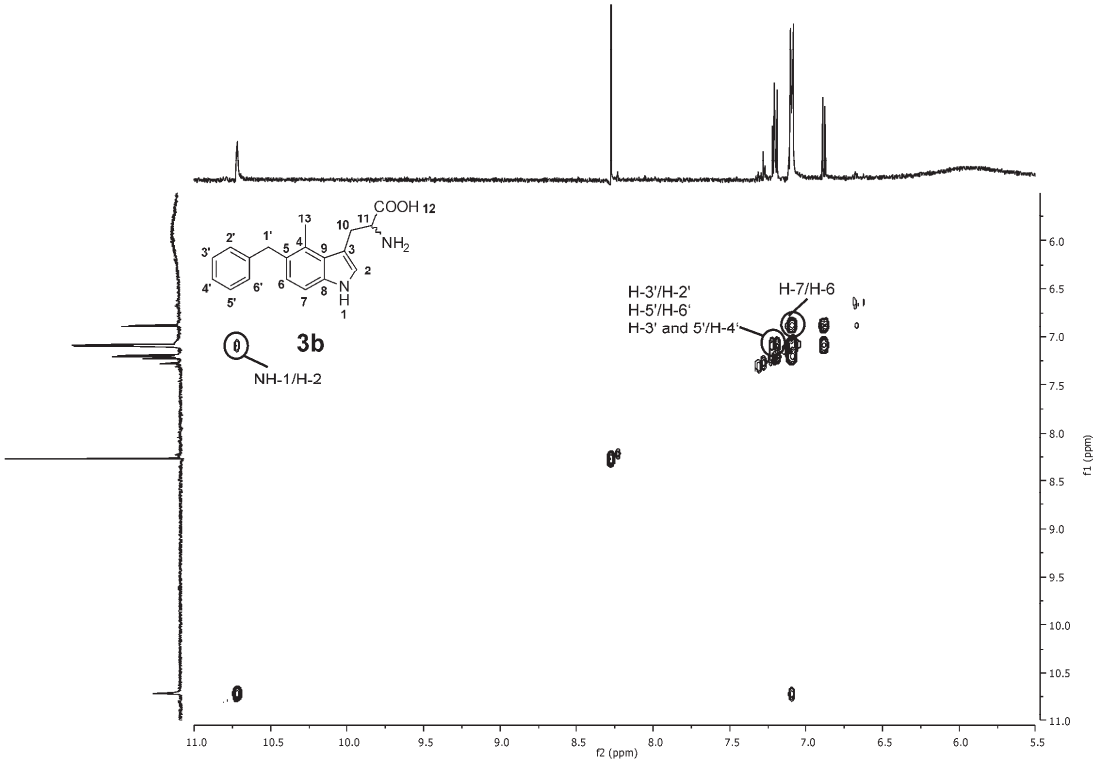


Figure S12. COSY spectrum of 5-benzyl-4-methyl-DL-tryptophan (**3b**) in DMSO-d6.

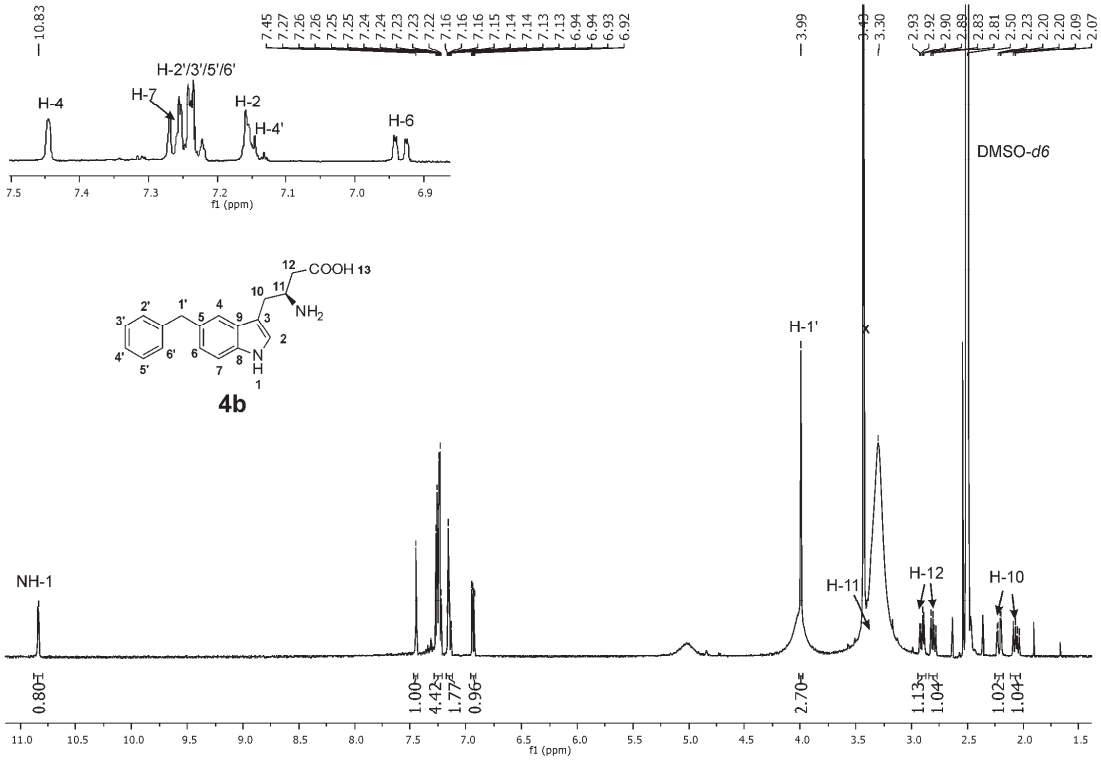


Figure S13. ^{1}H-NMR spectrum of 5-benzyl-L-β-homotryptophan (**4b**) in DMSO-d6.

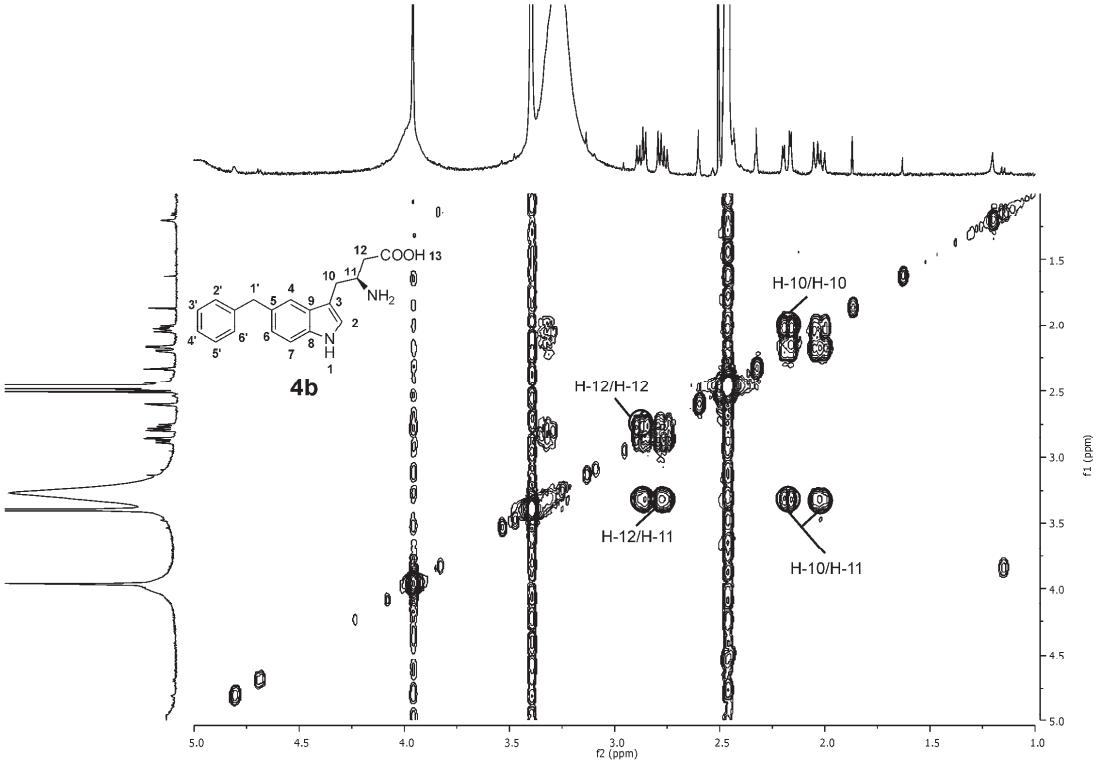


Figure S14. COSY spectrum of 5-benzyl-L-β-homotryptophan (**4b**) in DMSO-d6.

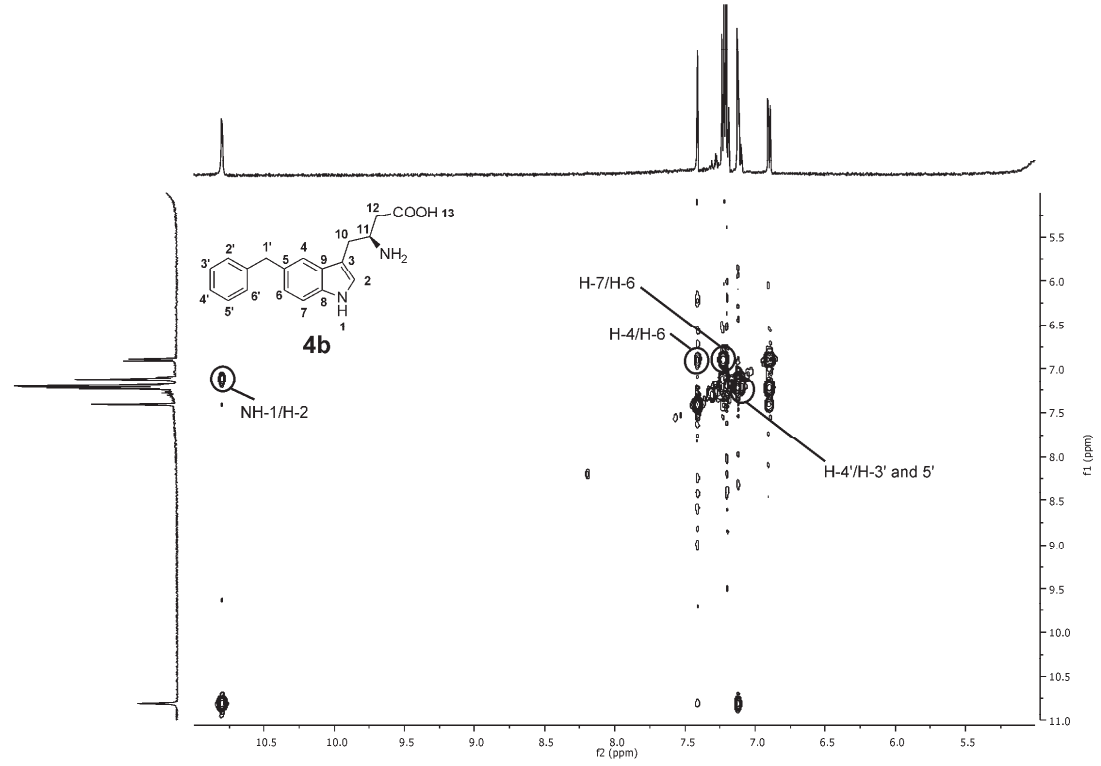


Figure S15. COSY spectrum of 5-benzyl-L-β-homotryptophan (**4b**) in DMSO-d6.

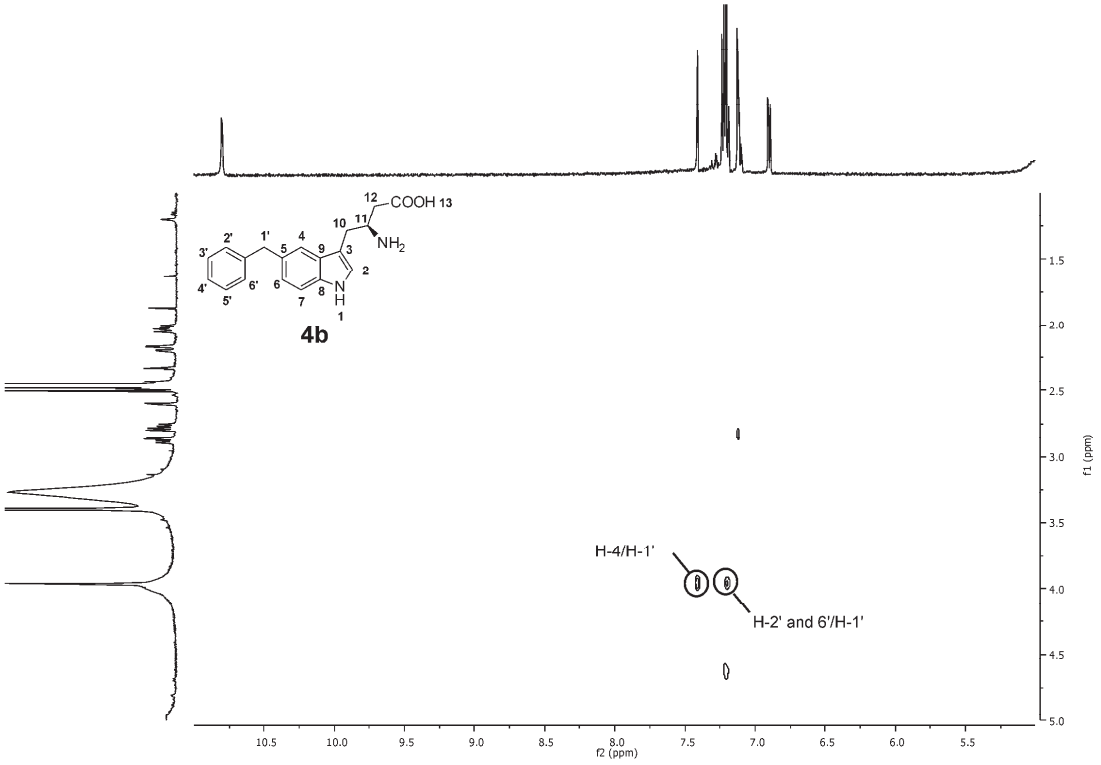


Figure S16. COSY spectrum of 5-benzyl-L-β-homotryptophan (**4b**) in DMSO-d6.

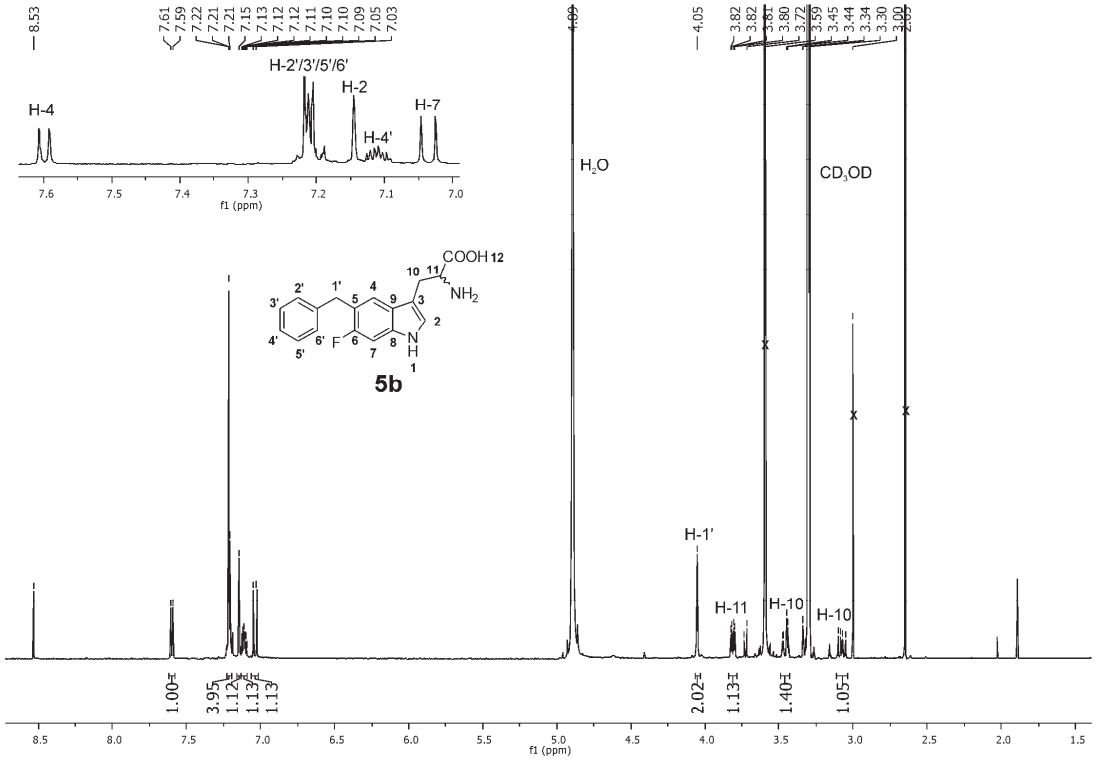


Figure S17. ^{1}H-NMR spectrum of 5-benzyl-6-fluoro-DL-tryptophan (**5b**) in CD_3OD.

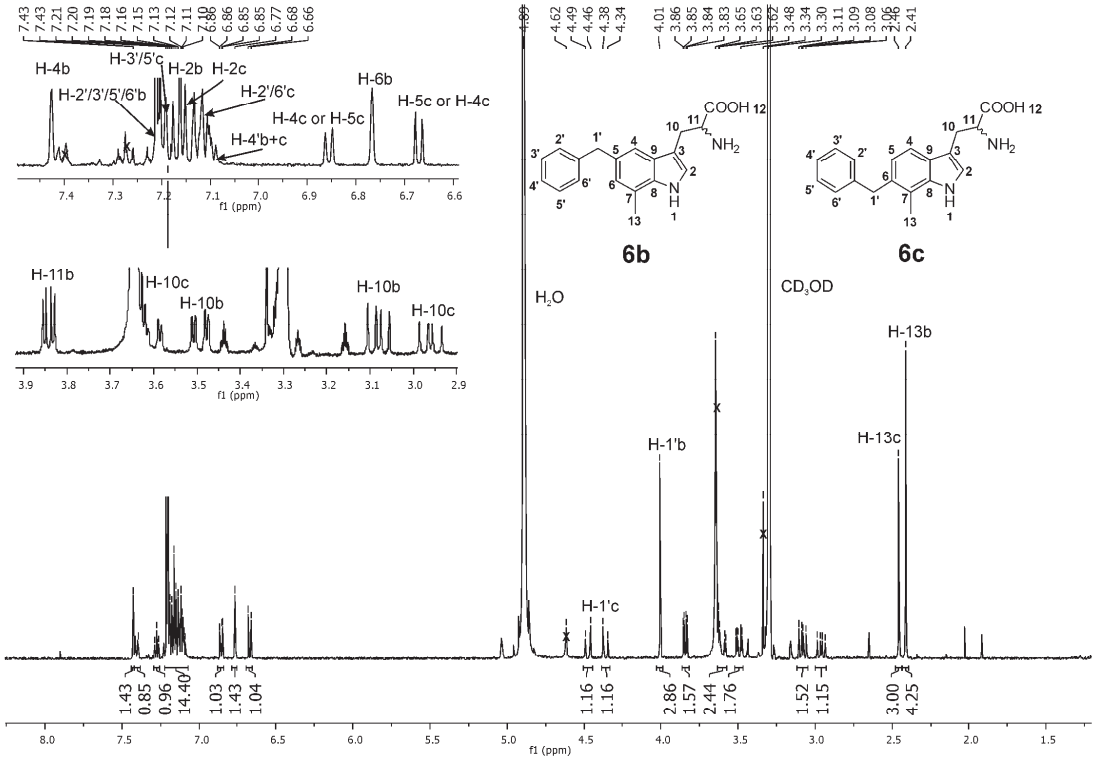


Figure S18. ^{1}H-NMR spectrum of 5-benzyl-7-methyl-DL-tryptophan (**6b**) and 6-benzyl-7-methyl-DL-tryptophan (**6c**) in CD_3OD.

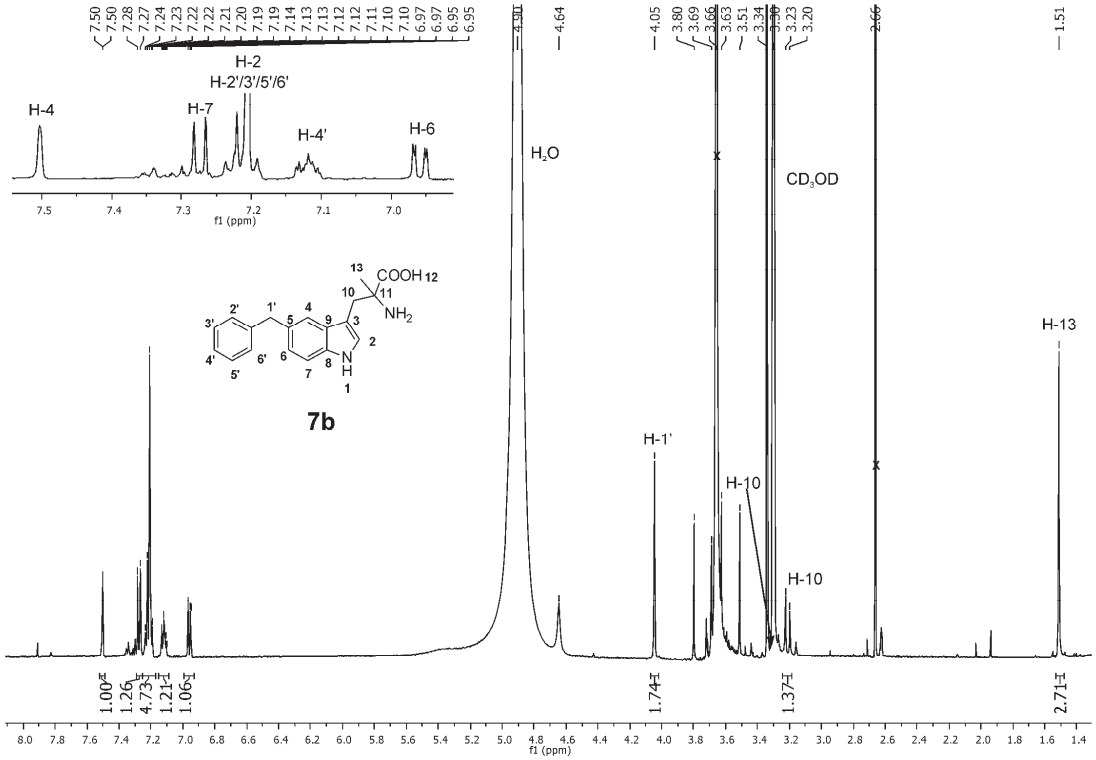


Figure S19. ^{1}H-NMR spectrum of 5-benzyl-α-methyl-DL-tryptophan (**7b**) in CD_3OD.

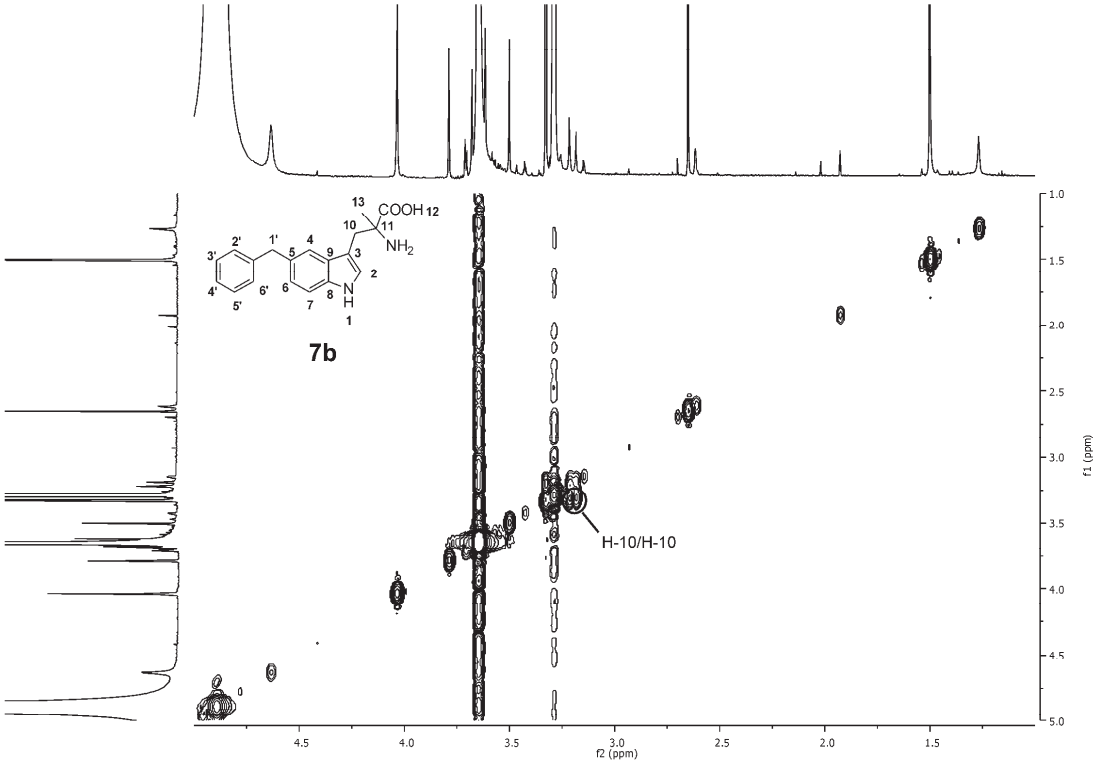


Figure S20. COSY spectrum of 5-benzyl-α-methyl-DL-tryptophan (**7b**) in CD_3OD.

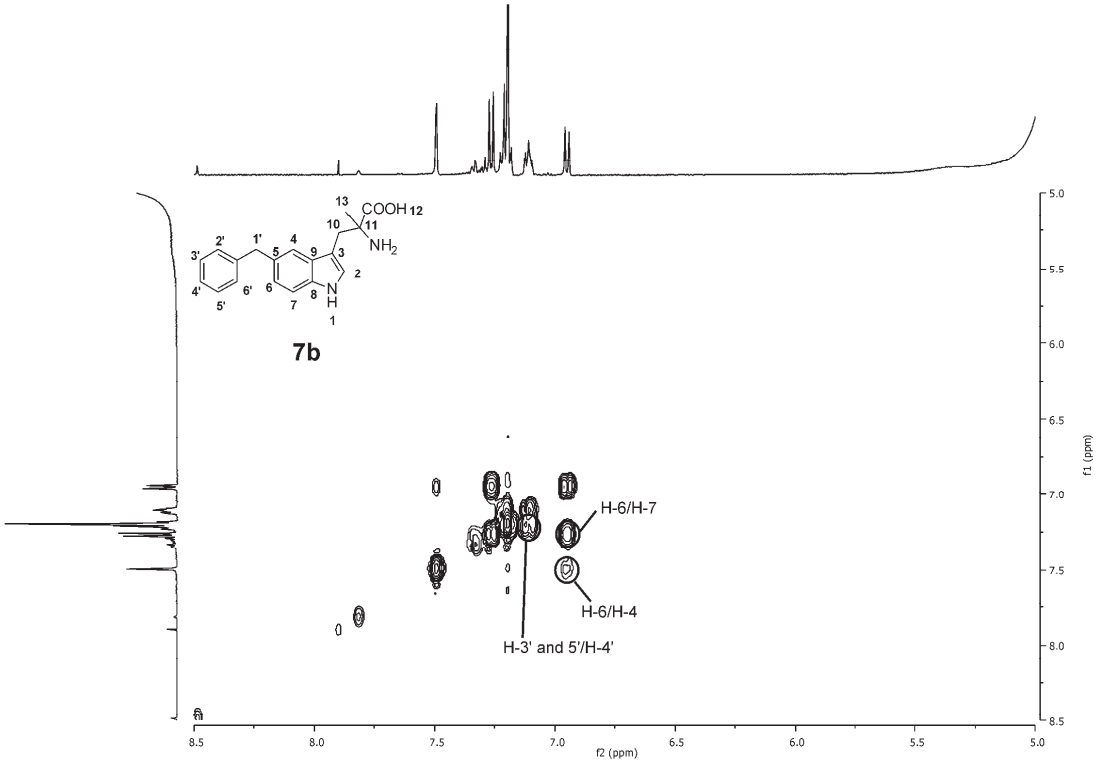


Figure S21. COSY spectrum of 5-benzyl-α-methyl-DL-tryptophan (**7b**) in CD_3OD.

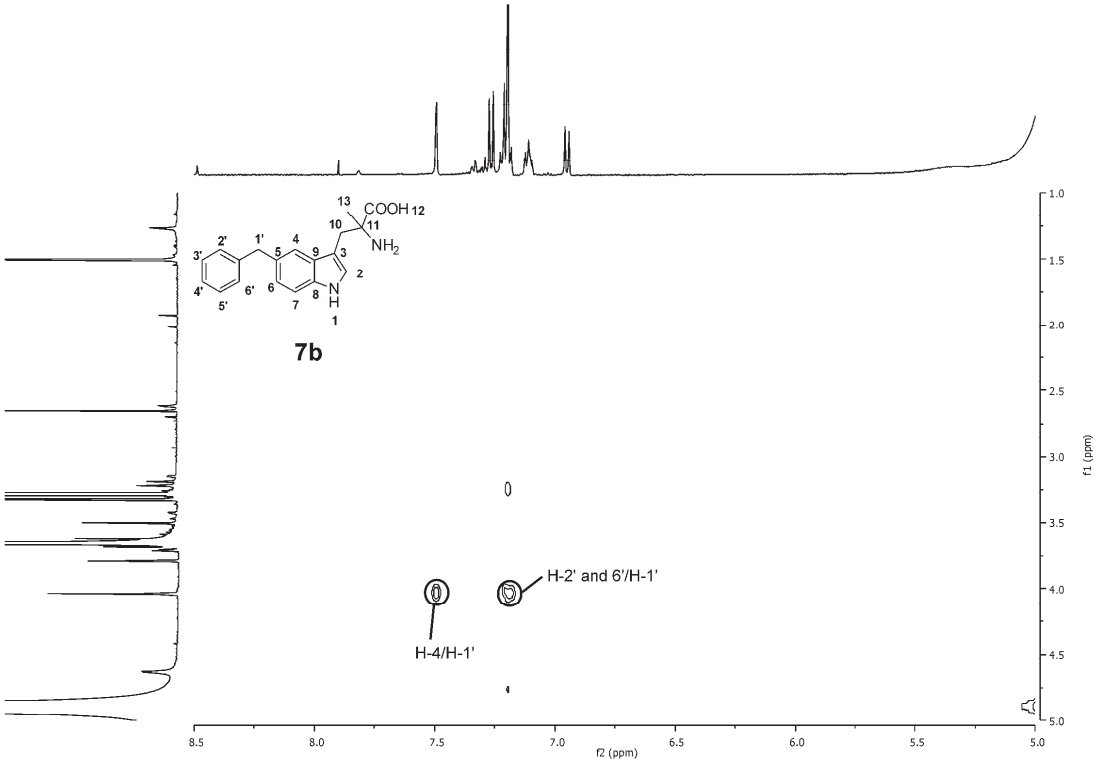


Figure S22. COSY spectrum of 5-benzyl-α-methyl-DL-tryptophan (**7b**) in CD_3OD.

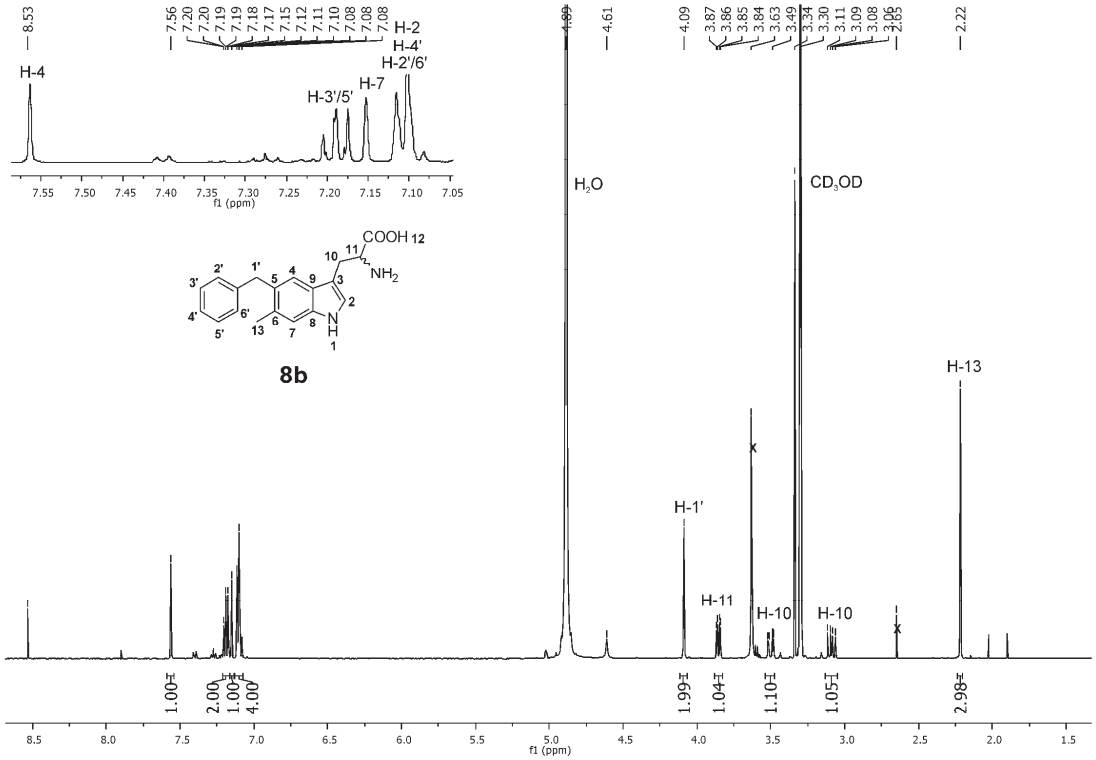


Figure S23. ^{1}H-NMR spectrum of 5-benzyl-6-methyl-DL-tryptophan (**8b**) in CD_3OD.

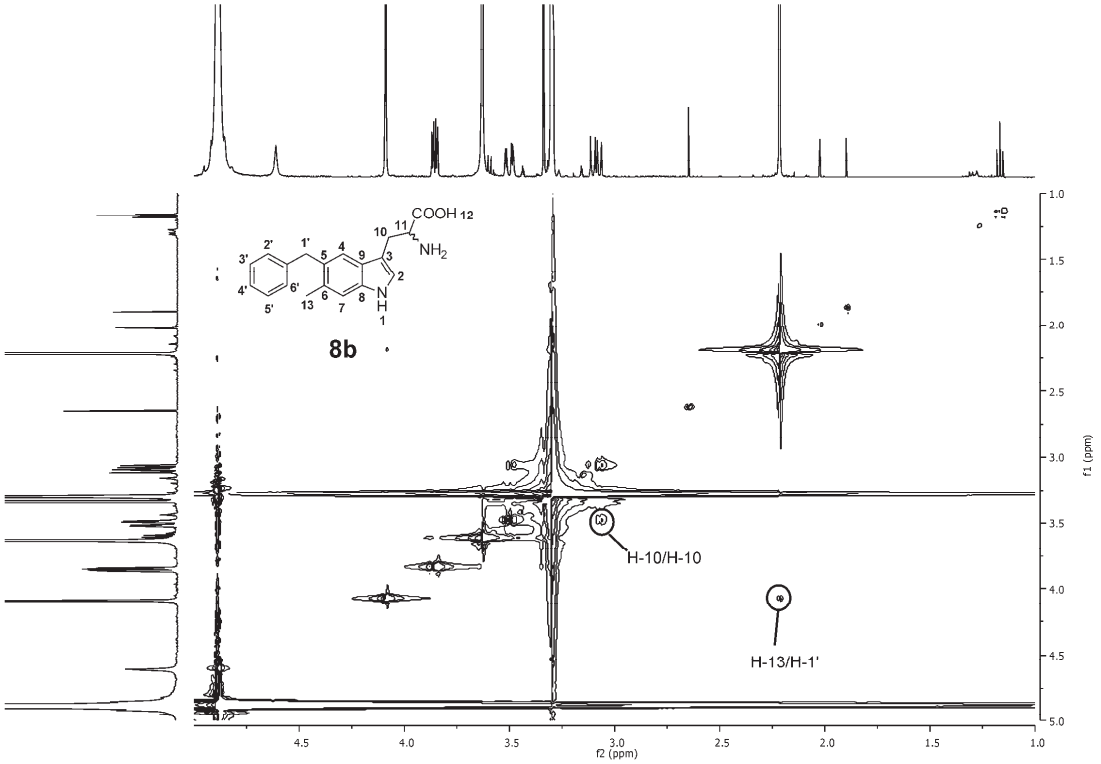


Figure S24. NOESY spectrum of 5-benzyl-6-methyl-DL-tryptophan (**8b**) in CD_3OD.

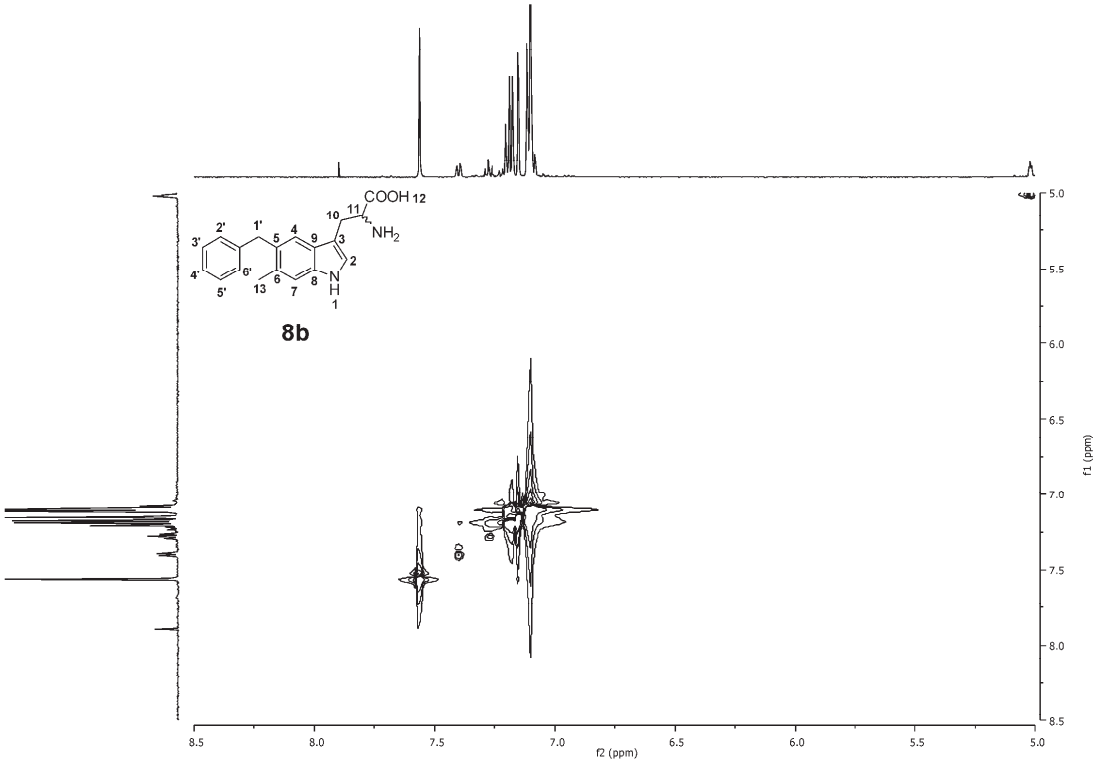


Figure S25. NOESY spectrum of 5-benzyl-6-methyl-DL-tryptophan (**8b**) in CD_3OD.

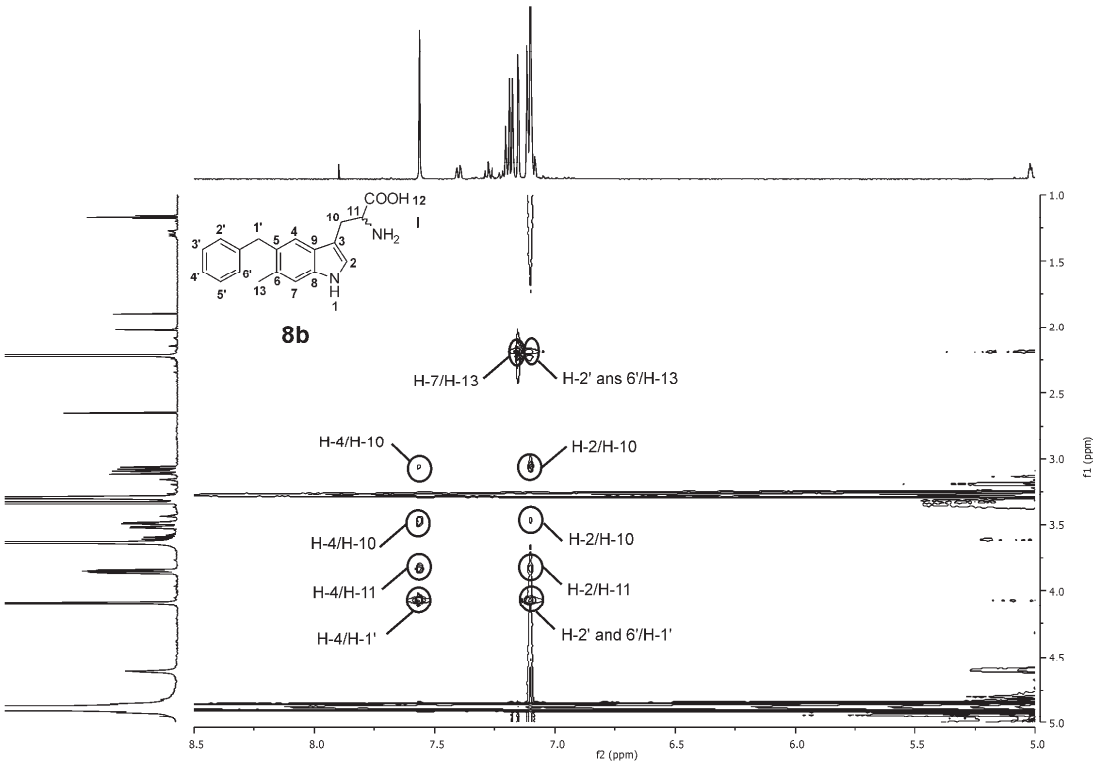


Figure S26. NOESY spectrum of 5-benzyl-6-methyl-DL-tryptophan (**8b**) in CD_3OD.

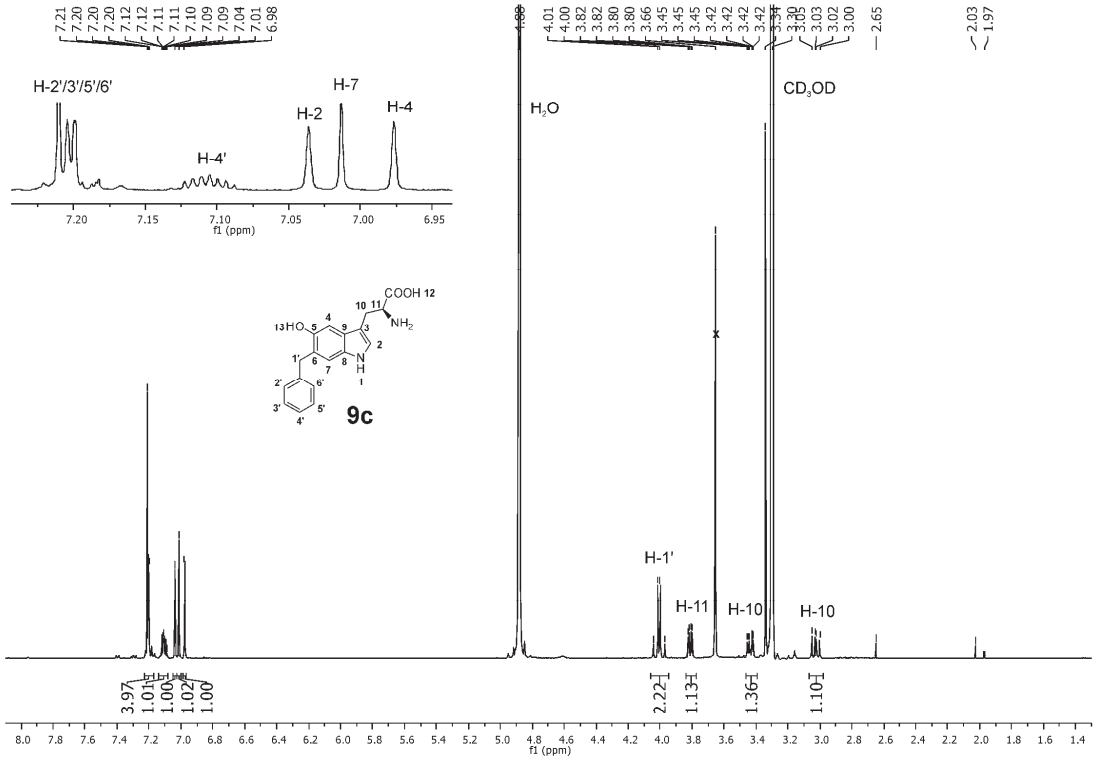


Figure S27. ^{1}H-NMR spectrum of 6-benzyl-5-hydroxy-L-tryptophan (**9c**) in CD_3OD.

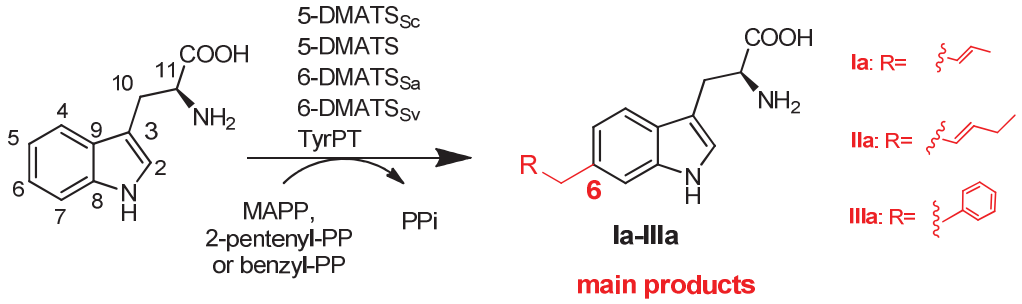
4.4. Unnatural DMAPP Analogues turn C-6 into the Preferable Alkylation/Benzylation Position for Tryptophan C5-, C6- and C7-Prenylating Enzymes

Unnatural DMAPP Analogues turn C-6 into the Preferable Alkylation/Benzylation Position for Tryptophan C5-, C6- and C7-Prenylating Enzymes

Julia Winkelblech,[†§‡] Mike Liebhold, [†‡] Xiulan Xie,[#] Shu-Ming Li[†§]*

[†]Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17A, 35037 Marburg, Germany; [§]Philipps-Universität Marburg, Zentrum für Synthetische Mikrobiologie, D-35032 Marburg, Germany; [#]Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany

Supporting Information Placeholder



ABSTRACT: The behaviors of two L-tryptophan *C5*-prenyltransferases 5-DMATS and 5-DMATS$_{Sc}$, two *C6*-prenyltransferases 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ and one L-tyrosine prenyltransferase with a tryptophan *C7*-prenyltransferase activity were investigated in the presence of two unnatural alkyl (MAPP, 2-pentenyl-PP) and one benzyl donor (benzyl-PP). Structure elucidation revealed the identification of *C6*-alkylated or benzylated derivatives as main products of these enzymes.

Secondary metabolites with biological activities represent an important source for medicinal research and drug development.[1,2] They are widely distributed in nature, especially in plants and microorganisms.[1-3] Among microorganisms, fungi of ascomycetes and bacteria of actinomycetes are important producers of biologically active compounds.[3,4] Due to significant progress in genome sequencing and genome mining, a number of gene clusters involved in the biosynthesis of such metabolites have been identified in recent years.[4-8] A large group of natural products comprises the prenylated aromatic substances derived from prenyl diphosphate and an aromatic scaffold from different pathways.[9,10] Prenyltransferases catalyze the linkage of these two residues and play an important role in the creation of structural diversity of these compounds. Indole prenyltransferases belong to the **di**methyl**a**llyl**t**ryptophan **s**ynthase (DMATS) superfamily, which catalyze the underlying prenylation reaction of indole derivatives in nature, and represent one of the most investigated class of prenyltransferases.[11] In the presence of the natural prenyl donor dimethylallyl diphosphate (DMAPP), most members of this superfamily usually show remarkable flexibility towards their aromatic substrates, but high regioselectivity of the prenylation position on the indole ring.[12-15] These characteristics were observed for fungal tryptophan prenyltransferases, e.g. FgaPT2, 5-DMATS and 7-DMATS from different *Aspergillus* spp., which catalyze tryptophan *C4*-, *C5*- and *C7*-prenylations, respectively. Two bacterial enzymes, SCO7467 from *Streptomyces coelicolor* A3(2) and IptA from *Streptomyces* sp. SN-593, are tryptophan *C5*- and *C6*-prenyltransferases, respectively.[12,16] Recently, two further 6-DMATS enzymes, 6-DMATS$_{Sa}$ (SAML0654) from *Streptomyces ambofaciens* (*S. ambofaciens*) ATCC238 and 6-DMATS$_{Sv}$ (Strvi8510) from *Streptomyces violaceusniger (S. violaceusniger)* Tü 4113 were identified and characterized biochemically. These two 6-DMATS enzymes showed high flexibility towards their prenyl donor and acceptor.[17] In contrast to other indole prenyltransferases, both DMAPP and geranyl diphosphate (GPP) were used by both enzymes.[17] Consequently, this flexibility makes them interesting candidates for further investigations on the acceptance of unnatural alkyl or benzyl donors.

Biochemical investigations on the tryptophan prenyltransferases FgaPT2 and 5-DMATS with monomethylallyl (MAPP) and 2-pentenyl diphosphate (2-pentenyl-PP) showed that these enzymes also accepted such unnatural alkyl donors. The alkylation positions were shifted partially or completely to the neighboring position.[18] The tryptophan *C4*-prenyltransferase FgaPT2 even accepted benzyl diphosphate (benzyl-PP) as substrate and catalyzed the regiospecific benzylation of L-tryptophan at position C-5.[19] Our previous data on the reactions of tryptophan prenyltransferases with unnatural alkyl and benzyl donors were limited to enzymes, which catalyzed the transfer reactions of dimethylallyl moiety onto position C-4 and C-5 of the indole ring.[18,19] In a previous study, the behavior of the 7-DMATS from *A. fumigatus* could not be investigated in detail, due to its low activity in the presence of unnatural DMAPP analogues.[18] After availability of the two tryptophan *C6*-prenyltransferases 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ as well as of the L-tyrosine prenyltransferase TyrPT with a tryptophan *C7*-prenyltransferase activity[20] in our laboratory, we initiated to prove their behavior towards MAPP, 2-pentenyl-PP and benzyl-PP.

The purified recombinant proteins 6-DMATS$_{SA}$ and 6-DMATS$_{Sv}$ were firstly incubated with L-tryptophan in the presence of one of the three unnatural DMAPP analogues MAPP (**I**), 2-pentenyl-PP (**II**), and benzyl-PP (**III**). HPLC analysis of the enzyme assays showed clear product formation in all of these reaction mixtures, with the highest conversions of 91.2% and 89.7% observed in the presence of 2-pentenyl-PP for 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$, respectively (Figure 1). Lower conversion yields of 51.4% and 37.8% were observed in the incubation mixtures with MAPP and 13.7% and 8% with benzyl-PP (Figure 1, Scheme 1, Table S1). To determine the alkylation position at **L**-tryptophan, enzyme assays were prepared in large scale. The enzyme products **Ia-IIIa** were isolated from both assays of 6-DMATS$_{SA}$ and 6-DMATS$_{Sv}$ on HPLC and their structures were elucidated by MS and NMR

analyzes. For better understanding, we named the products by a combination of **I** (product from MAPP), **II** (2-pen-PP) or **III** (benzyl-PP) with **a** (regular alkyl or benzyl at C-6), **b1** (regular alkyl at C-7), **b2** (reverse alkyl at C-7), **b** (benzyl at C-7) or **c** (regular alkyl or benzyl at C-5).

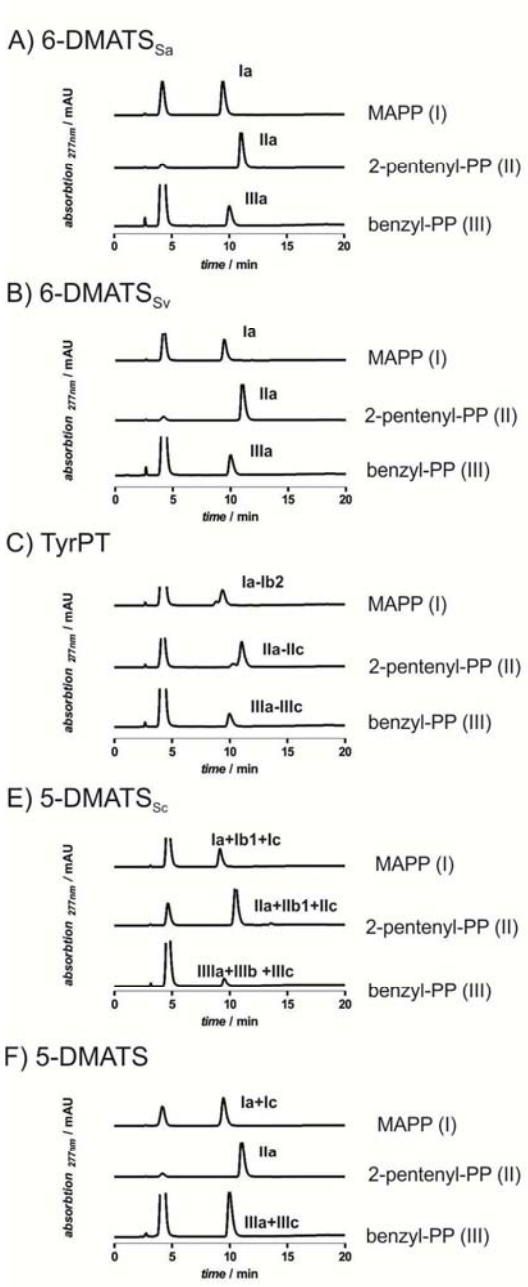


Figure 1. HPLC analysis of the reaction mixtures of L-tryptophan with unnatural DMAPP analogues

MS data confirmed the monoalkylation or benzylation of the isolated products (Table S2). ^{1}H-NMR analysis (For structural elucidation see Supporting Information) proved the regular attachment of the alkyl or benzyl residue onto position C-6 of the indole ring in all of these cases (Table S4, Figure S9-S10). This conclusion was drawn by comparison of the coupling patterns of the signals for aromatic protons with those of the published data for *C6*-alkylated L-tryptophan.[17-19] In the presence of the natural prenyl donors DMAPP or GPP, 6-DMATS$_{SA}$ and 6-DMATS$_{Sv}$ also catalyzes a C-6 prenylation.[17] Therefore, the alkylation position for both enzymes was proven to be independent of the used alkyl or benzyl donor.

Taking the data on 6-DMATS$_{SA}$ and 6-DMATS$_{Sv}$ with the previous published results on FgaPT2 and 5-DMATS[18,19,21] together, we have shown the behavior of tryptophan *C4*-, *C5*- and *C6*-prenyltransferases towards unnatural DMAPP analogues. It would be interesting to complete this series with *C7*-prenylating enzymes. A previous study showed that the tryptophan *C7*-prenyltransferase 7-DMATS from *A. fumigatus*[22] accepted very poorly MAPP and 2-pentenyl-PP.[18] Recently, CAK41583 from *A. niger* was identified as a tyrosine prenyltransferase (TyrPT), catalyzing an O-prenylation at the phenolic hydroxy group of L-tyrosine.[20] As in the case of SirD from *Leptosphaeria maculans*,[23] TyrPT also catalyzed the transfer reaction of a dimethylallyl moiety from DMAPP to C-7 of L-tryptophan and several derivatives thereof.[20] The broad substrate specificity of TyrPT led us to test its activity for DMAPP analogues in the presence of L-tryptophan. In analogy to 6- DMATS$_{SA}$ and 6-DMATS$_{Sv}$, TyrPT was incubated with L-tryptophan in the presence of MAPP, 2-pentenyl-PP and benzyl-PP. Product formation was detected in all three incubation mixtures (Figure 1). However, the observed enzyme activities were much lower than those of the two 6-DMATS enzymes. Total product yields of 37.8, 17.5 and 7.8% were calculated for 2-pentenyl-PP, MAPP and benzyl-PP, respectively (Figure 1, Scheme 1, Table S1). This is justified by the fact that L-tyrosine, but not L-tryptophan is the best accepted aromatic substrate by TyrPT, also in the presence of DMAPP. Interestingly, the ratio of the relative activities towards the three DMAPP analogues was similar to those of the two 6-DMATS enzymes. In contrast to the unique *C7*-prenylation of L-tryptophan by TyrPT in the presence of DMAPP, interpretation of the ^{1}H-NMR spectra of the isolated peaks indicated the presence of more than one product in the incubation mixtures with DMAPP analogues. Optimization of the HPLC conditions and the application of a Chiralpak Zwix (+) column (Supporting Information) allowed a partial separation of these product mixtures.

HPLC analysis of the incubation mixture with 2-pentenyl-PP and interpretation of the NMR data led to the identification of three substances with a regular alkyl moiety attached to C-6 (**IIa**), C-7 (**IIb1**) and C-5 (**IIc**), respectively. Product yields of 21.5, 9.9 and 3.3% were calculated for these products (Scheme 1, Supporting Information). In addition, a reversely *C7*-alkylated L-tryptophan (**IIb2**) was isolated with a product yield of 3.0% (Scheme 1, Figure S21). With MAPP as alkyl donor, regularly *C6*- (**Ia**) and *C7*-alkylated (**Ib1**) as well as reversely *C7*-alkylated derivatives (**Ib2**) were identified by interpretation of their NMR-spectra (Supporting Information). Product yields of 8.6, 5.7 and 3.2% were calculated for **Ia**, **Ib1** and **Ib2**, respectively. Regularly *C6*-, *C7*- and *C5*-benzylated products (**IIIa, IIIb, IIIc**) with product yields of 6.7, 1.5 and 1.1% were identified in the reaction mixture of L-tryptophan with benzyl-PP. These results demonstrated clearly that *C6*-alkylated or benzylated derivatives were predominant products of TyrPT reactions in the presence of the unnatural donors (Scheme 1) and differed clearly from that of L-tryptophan with DMAPP.[20]

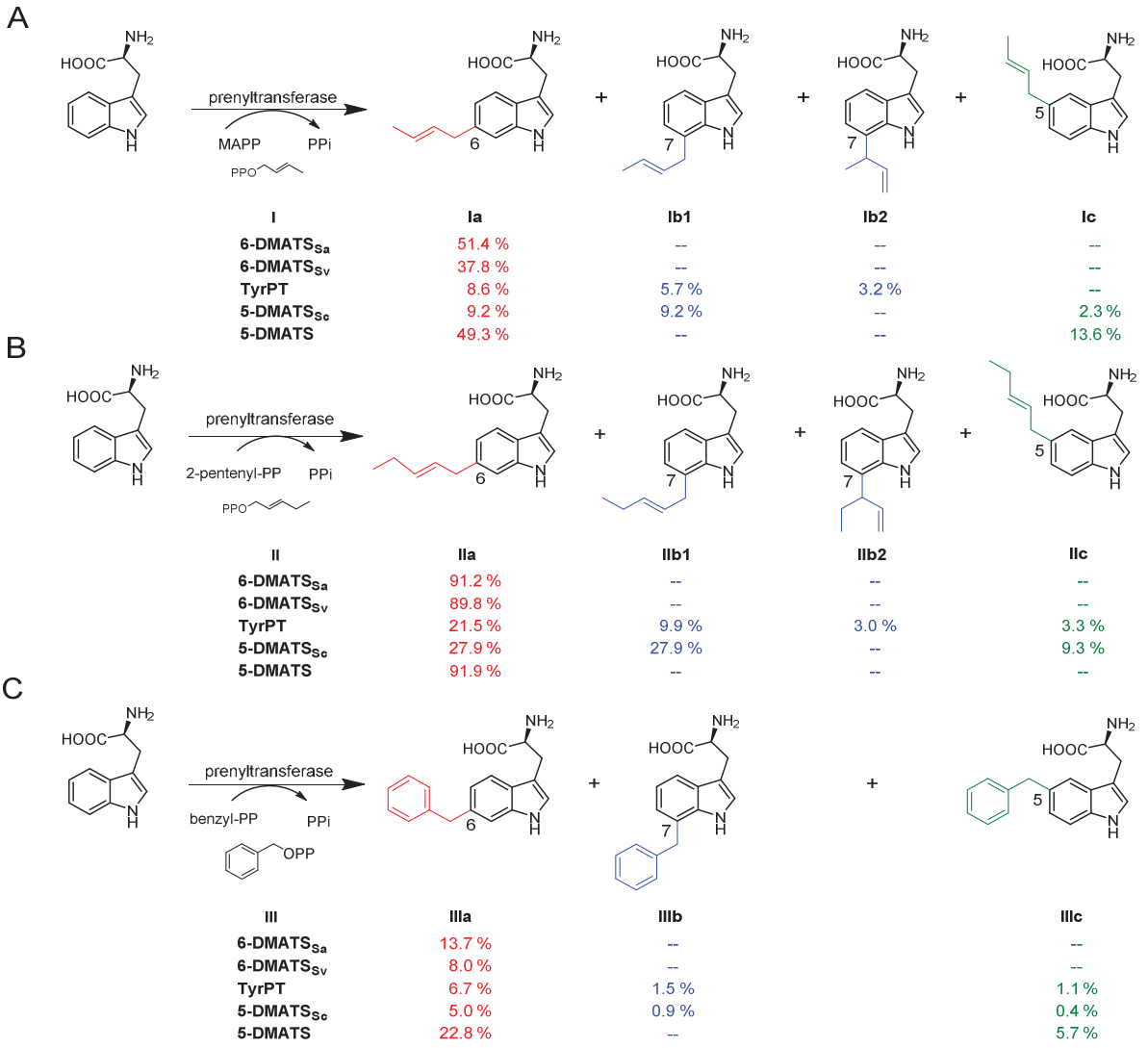
As described above, the two 6-DMATS enzymes from bacteria catalyzed regiospecific alkylation and only one product with the same position, i.e, C-6, was identified, independent of DMAPP, GPP[17] or DMAPP analogues MAPP, 2-pentenyl-PP or benzyl-PP. In comparison, the fungal prenyltransferases FgaPT2 and 5-DMATS catalyzed regiospecific prenylation in the presence of DMAPP.[13,14] But in the presence of the unnatural DMAPP analogues, the regioselectivity was partially or completely shifted.

[18,19] In the presence of L-tryptophan, the fungal L-tyrosine O-prenyltransferase TyrPT also accepted DMAPP analogues as substrates, but catalyzed the formation of *C6*- instead of *C7*-alkylated or benzylated products as predominant products. These results raised an important question. It seems that in the presence of DMAPP analogues, C-6 is the preferable alkylation position for enzymes, which usually catalyzed the prenylation of L-tryptophan at C-5 (like 5-DMATS), C-6 (6-DMATS enzymes) and C-7 (TyrPT). However, it cannot be excluded that the observed regio-specific alkylation or benzylation of L-tryptophan at the same position by 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ in the presence of DMAPP, GPP and DMAPP analogues is based on their bacterial origin. 5-DMATS and TyrPT are fungal enzymes and therefore showed different behaviors. The later hypothesis would be also supported by the fact that FgaPT2 catalyzed tryptophan alkylation and benzylation in the presence of these unnatural alkyl and benzyl donors with partial or complete shift of attachment positions.[18,19]

To clarify the possible reason for this difference, the tryptophan prenyltransferase SCO7467 from the bacterium *Streptomyces coelicolor* A3(2), which functions as a 5-DMATS (5-DMATS$_{Sc}$) in the biosynthesis of 5-dimethylallylindole-3-acetonitrile,[16,24] was overproduced in *E. coli* as reported by Ozaki,[24] purified and investigated in the presence of MAPP, 2-pentenyl-PP and benzyl-PP. For comparison, the behavior of the fungal 5-DMATS from *A. clavatus*,[13] towards MAPP and 2-pentenyl-PP[18] was reproduced in this study. In addition, this enzyme was assayed with benzyl-PP in the presence of L-tryptophan.

The previously reported data for 5-DMATS were very well reproduced in this study by identification of *C5*- and *C6*-alkylated products with MAPP, with products yields of 13.6 and 49.3%, respectively. In the presence of 2-pentenyl-PP, the alkylation position was completely shifted from C-5 to C-6. Similar to those of MAPP, *C5*- and *C6*-benzylated products with yields of 5.7 and 22.8% were detected in the assay with benzyl-PP (Figure 1, Scheme 1). Again, *C6*-alkylated or benzylated L-tryptophan represented the predominant product.

Scheme 1. Alkylation/benzylation of L-tryptophan catalyzed by *C5*-, *C6*- and *C7*-prenylating enzymes in the presences of DMAPP analogues



6-DMATS$_{Sa}$: 6-DMATS from *S. ambofaciens*
6-DMATS$_{Sv}$: 6-DMATS from *S. violaceusniger*
5-DMATS: 5-DMATS from *A. clavatus*
5-DMATS$_{Sc}$: 5-DMATS from *S. coelicolor*
TyrPT: from *A. niger* with a C7-prenyltransferase activity
-- product yields < 0.3%,

HPLC analysis clearly revealed product formation in the reaction mixtures of L-tryptophan with the recombinant 5-DMATS$_{Sc}$ in the presence of all three DMAPP analogues (Figure 1). HR-MS data confirmed the attachment of one alkyl or benzyl residue on the substrate for all of the obtained products (Table S2). Structure elucidation by NMR indicated that the isolated product peaks consisted of more than one substance. *C6*-, *C7*- and *C5*-alkylated derivatives were identified with ratios of 3:3:1 for 2-pentenyl-PP and of 4:4:1 for MAPP. By using a Chiralpak Zwix (+) column, the *C7*-alkylated products were purified from these mixtures (Scheme 1, Figure S1, Table S4-S6). With 2-pentenyl-PP as alkyl donor, a product yield of 9.3% was calculated for *C5*-(**IIc**) and 27.9% each for *C6*-(**IIa**) and *C7*-(**IIb1**) alkylated L-tryptophan (Scheme 1). In the case of MAPP, product yields of 9.2, 9.2 and 2.3% were determined for **Ia**, **Ib1** and **Ic**, respectively. Inspection of the NMR spectra of the products obtained with benzyl-PP revealed the presence of 6-benzyl-L-tryptophan (**IIIa**) with a product yield of 5.0 % and 7-benzyl-L-tryptophan (**IIIb**) of 0.9% (Figure S11, S22). In addition, signals of a *C5*-benzylated L-tryptophan (**IIIc**) with a product yield of 0.4% could also be observed (Figure S25).

Determination of the kinetic parameters of the enzymes with the DMAPP analogues indicated that the observed reactions were consistent with Michaelis-Menten-kinetics. K_M values in the range of 0.011 to 0.13 mM proved their relatively high affinity towards the DMAPP analogues. In contrast, the turnover numbers of the reactions with these DMAPP analogues were much lower than those with DMAPP. As observed in Figure 1 and given in Scheme 1, 2-pentenyl-PP was accepted in most cases as the best unnatural alkyl donor.

In conclusion, all the tested enzymes used MAPP, 2-pentenyl-PP and benzyl-PP as substrates and catalyzed Friedel-Crafts alkylation or benzylation on the indole ring. The observed reactions differ from each other in relative activities and regioselectivity of the attached position and therefore one to four alkylated or benzylated derivatives have been identified as enzyme products (Scheme 1). From Scheme 1, it is obvious that in the presence of unnatural DMAPP analogues, C-6 of tryptophan was the preferable alkylation and benzylation position for tryptophan *C5*-, *C6*- and *C7*-prenylating enzymes. *C6*-alkylated or benzylated derivatives were identified in all the reaction mixtures. It was found as unique product in the cases of the both 6-DMATS enzymes with all of the three DMAPP analogues or as one of two main products in the reaction mixtures of 5-DMATS$_{Sc}$ with MAPP and 2-pentenyl-PP. Such derivatives were clear predominant products in all other reaction mixtures. From Scheme 1, it is also clear that the tryptophan *C5*-prenyltransferases 5-DMATS and 5-DMATS$_{Sc}$ as well as TyrPT with a tryptophan *C7*-prenyltransferase activity also produced *C5*- or/and *C7*-alkylated or benzylated derivatives, indicating a shift of the alkylation or benzylation position from C-5 to C-7 and *vice versa*. The preference of C-6 for these enzymes in the presence of the unnatural DMAPP analogues could be explained by the fact that this position would provide more space for an attack of the alkyl or benzyl carbocation. Further structural analysis of these enzymes in complex with unnatural DMAPP analogues would provide new insights into the substrate binding sites and give a more accurate explanation for this interesting phenomenon.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, HR-MS, NMR data and spectra are available free of charge via Internet at http://pubs.acs.org.and

Corresponding Author

* E-mail: shuming.li@staff.uni-marburg.de

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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Unnatural DMAPP Analogues turn C-6 into the Preferable Alkylation/Benzylation Position for Tryptophan C5-, C6- and C7-Prenylating Enzymes

Supporting Information

Julia Winkelblech,[†§‡] Mike Liebhold,[†‡] Xiulan Xie,[#] Shu-Ming Li[†§*]

[†]Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Deutschhausstrasse 17A, 35037 Marburg, Germany; [§]Philipps-Universität Marburg, Zentrum für Synthetische Mikrobiologie, D-35032 Marburg, Germany; [#]Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse, 35032 Marburg, Germany

[‡]these authors contributed equally to this work

Experimental Section

Chemicals

Synthesis of monomethylallyl-PP (MAPP), 2-pentenyl-PP and benzyl-PP was carried out as described previously.[1,2] L-tryptophan was purchased from Roth (Karlsruhe, Germany).

Overproduction and purification of the recombinant proteins

Gene expression and subsequent protein purification of the recombinant 6-DMATS$_{Sa}$-His$_6$, His$_8$-6-DMATS$_{Sv}$, His$_6$-TyrPT, 7-DMATS-His$_6$ and 5-DMATS-His$_6$ were carried out as described previously.[3-6]

Cloning and expression of 5-DMATS$_{Sc}$ (SCO7467)

PCR amplification of *SCO7467* from *Streptomyces coelicolor* A3(2) was carried out as described by Ozaki et al.[7] The expression vector pHis$_8$ containing the coding sequence was termed pML10. For optimal protein yield *E. coli* BL21 [DE3] cells harboring pML10 were cultivated in 1 L liquid lysogeny broth (LB) medium supplemented with kanamycin (50 µg ml^{-1}) till an OD$_{600}$ of 0.6. For induction of gene expression IPTG was added to a final concentration of 0.5 mM. After further incubation at 30°C and 220 rpm for 6 h, the recombinant protein was purified as routinely on Ni-NTA agarose.

Assays for determination of the enzyme activities

The reaction mixtures (100 µl) for determination of the enzyme activities contained 1 mM L-tryptophan, 5 mM $CaCl_2$, 2 mM alkyl-diphosphate (DMAPP, MAPP (**I**), 2-pentenyl-PP (**II**)) or benzyl-PP (**III**), 1.0-1.5% (v/v) glycerol, 50 mM Tris-HCl (pH 7.5) and 7.5 µM of purified recombinant protein. The reaction mixtures were incubated at 37 °C for 16 h. For HPLC

analysis on a RP-18 column, the reactions were terminated with 100 µL MeOH. Protein was then removed by centrifugation at 17.000 × g for 15 min.

Enzyme assays for isolation and structure elucidation

Assays for isolation of the enzyme products were carried out in large scales (10 mL) (1 mM L-tryptophan, 2 mM MAPP, 2-pentenyl-PP or benzyl-PP, 5 mM $CaCl_2$, 0.0%-1.5% (v/v) glycerol, 50 mM Tris-HCl (pH 7.5)) with 2 to 4 mg of purified recombinant protein. After incubation for 16 h at 37 °C, the reaction mixtures were terminated with 10 mL MeOH and precipitated protein was removed by centrifugation at 4.750 × g for 15 min. The obtained supernatant was then concentrated on a rotating vacuum evaporator.

HPLC analysis and isolation of the enzymatic products for structure elucidation

The enzyme products were analyzed on an Agilent series 1200 HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) with a Multospher 120 RP-18 column (250 × 4 mm, 5 µm, C+S-Chromatography Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1}. Water (solvent A) and methanol (solvent B) were used as solvents for analysis and isolation of the enzyme products. For analysis of the alkylated tryptophan, a linear gradient of 40-100% (v/v) solvent B over 15 min was used. The column was then washed with 100% solvent B for 5 min and equilibrated with 40% solvent B for 5 min. Detection was carried out on a photo diode array detector.

By using the same HPLC equipment and a semipreparative Multospher 120 RP-18 column (250 × 10 mm, 5 µm, C+S-Chromatographie Service, Langerwehe, Germany), the enzyme products were isolated at a flow rate of 2.5 mL min^{-1} and a gradient of 60-100% solvent B in 20-25 min. If necessary an isocratic step solvent B before the gradient was included for 5 min.

After each run the column was washed with 100% solvent B and equilibrated with 60% solvent B for 5 min.

A much better separation of the L-tryptophan derivatives with different alkylation positions on their indole ring was achieved by using a Chiralpak Zwix column (+) (150 × 3 mm, 3 µm, Chiral technologies Europe, Daicel Group, Illkirch Cedex, France). This column was used for detailed investigations on the enzyme products in the incubation mixtures and for separation of the product mixtures, which were isolated previously by using the reported semipreparative Multospher 120 RP-18 column. Analysis of the enzyme assays and isolation of the products were carried out at a flow rate of 0.5 mL min^{-1} with water (solvent A) and methanol (solvent B) as solvents. 50% solvent B was used in an isocratic run.

NMR and MS analyses as well as structure elucidation

NMR including two-dimensional HSQC and HMBC spectra were recorded on a JEOL ECA-500 500 MHz spectrometer (JEOL Germany GmbH, Munich, Germany) or Bruker Avance 600 MHz spectrometer (Bruker Corporation, Billerica, USA), respectively. Chemical shifts were referred to the signals of CD_3OD at 3.31 ppm. The spectra were processed with MestReNova 6.0.2. The isolated compounds were also analyzed by electrospray ionization or electron impact mass spectrometry (ESI-MS, EI-MS) on a Q-Trap 2000 (Life Technologies Ltd, Paisley PA4 9RF, United Kingdom) and high resolution electrospray ionization mass or electron impact mass spectrometry (HR-EI-MS, HR-ESI-MS) on an Auto SPEC (Waters MS Technology Centre, Manchester, United Kingdom).

In the ^{1}H-NMR spectra of **Ia**, signals of the indole moiety at δ_H 7.60 (1H, dd, 8.1, 0.4), δ_H 7.15 (1H, d, 0.7), δ_H 7.12 (1H, s) and δ_H 6.89 (1H, dd, 8.1, 1.4) superimposed with those for H-4, H-7, H-2 and H-5 of 6-monomethylallyl-L-tryptophan, respectively.[1] The signals of H-10 and H-11 as well as H-1’ and H-4’ of **Ia** were also overlapping almost completely (maximum

shift 0.03 ppm) with those of 6-monomethylallyl-L-tryptophan.[1] This proved unequivocally the regular C6-alkylation of L-tryptophan with MAPP as alkyl donor in the presence of the tested prenyltransferases (6-DMATS$_{Sa}$, 6-DMATS$_{Sv}$, TyrPT, 5-DMATS$_{Sc}$ and 5-DMATS). Comparison of the ^{1}H-NMR spectra of **IIa** with that of C6-(2-pentenyl)-L-tryptophan[1] showed nearly identical chemical shifts and coupling patterns for all of the protons. This verified the regular alkylation of L-tryptophan at position C-6 of the indole ring by using 2-pentenyl-PP as alkyl donor. The aromatic protons of **IIIa** at δ_H 7.60 (1H, dd, 8.2, 0.6), δ_H 7.18 (1H, s), δ_H 7.12 (1H, s) and δ_H 6.93 ppm (1H, dd, 8.2, 1.5) showed the same coupling pattern and chemical shifts as observed for **Ia** and **IIa**. These signals also corresponded to those of C6-alkylated L-tryptophan derivatives[1] and therefore proved the *C6*-benzylation of L-tryptophan. The chemical shifts observed for H-10 and H-11 at 3.48 ppm, 3.11 ppm and 3.82 ppm, also overlapped very well with those of the other C6-alkylated L-tryptophan derivatives. The five additional aromatic protons and two additional aliphatic protons observed in the ^{1}H-NMR spectra of **IIIa** confirmed the presence of the benzyl moiety.

From the incubation mixtures of TyrPT and 5-DMATS$_{Sc,}$ the regular alkylated products **Ib1** and **IIb1** as well as the regular benzylated product **IIIb** were isolated. The ^{1}H-NMR spectrum of **IIb1** showed one singlet at 7.19 ppm, two doublets at 7.55 and 6.93 ppm and one triplet at 7.00 ppm in the aromatic region of the spectrum. This indicated an alkylation at position C-4 or C-7 of the indole ring. In the HMBC spectrum of **IIb1** (Figure S16-S19), correlations between H-10 at 3.13 ppm and C-2 at 124.7 ppm, C-11 at 56.5 ppm with two quaternary carbon atoms at 109.9 ppm and 128.4 ppm were observed. Correlations between the proton at 7.00 ppm, which is either H-5 or H-6, and two quaternary carbon atoms at 128.4 ppm and at 125.1 ppm but not with that at 109.9 ppm were detected. Consequently, the quaternary carbons at 109.9 ppm and 128.4 ppm were assigned to C-3 and C-9, respectively. Therefore, the signal at 7.00 ppm was assigned to H-5. Further correlations were found between the doublet at 7.56 ppm and the quaternary carbons C-3, C-9 and another one at 136.8 ppm.

These correlations are only possible, if the proton at 7.56 ppm is for H-4 and the carbons at 136.8 ppm and 125.1 ppm are for C-8 and C-7, respectively. Thus, an alkylation at position C-7 was proven. This was further confirmed by correlations between H-1' at 3.54 ppm and the quaternary carbons at 125.1 ppm and 136.8 ppm, but not with that at 128.4 ppm (C-9). In addition, the doublet at 6.93 ppm also correlated with the quaternary carbons at 125.1 ppm and 136.8 ppm as well as the signal for H-1'. The signals at 125.1 ppm, 136.8 ppm and 6.93 ppm were assigned to C-7, C-8 and H-6, accordingly.

The chemical shifts of the tryptophan moiety of **Ib1** at 7.56 (H-4), 7.19 (H-2), 7.00 (H-5), 6.93 (H-6), 3.85 (H-11), 3.51 (H-10) and 3.14 ppm (H-10) almost completely overlapped with those of **IIb1**. Similar spectrum was obtained for **IIIb**. Therefore, the alkylation position of **Ib1** and benzylation position of **IIIb** were assigned unequivocally to C-7 of the indole ring.

In the presence of TyrPT, the additional products **Ib2** and **IIb2** were found by using MAPP and 2-pentenyl-PP as alkyl donor, respectively. The coupling patterns and chemical shifts of the aromatic protons of both compounds corresponded very well to those of **Ib1** and **IIb1**, confirming a C7-alkylation of L-tryptophan (Slight shifts of approximate 0.05 ppm were observed). However, the signals of the alkyl residues of **Ib2** and **IIb2**, displayed distinct chemical shifts and coupling patterns in comparison to those of **Ib1** and **IIb1**. The coupling pattern for H-1' at δ_H 5.12 (1H, dt, 17.3, 1.7) and 5.04 ppm (1H, dt, 10.3, 1.7) as well as for H-2' at δ_H 6.12 ppm (1H, ddd, 17.3, 10.3, 6.3) in the spectrum of **Ib2** showed clearly a reverse alkylation.[9] The same was true for H-1' at δ_H 5.08 (1H, dt, 17.2, 1.5) and δ_H 4.99 ppm (1H, ddd, 10.2, 1.9, 1.0) as well as for H-2' at δ_H 6.06 ppm (1H, ddd, 17.2, 10.2, 7.6) in the spectrum of **IIb2**. This proved the reverse orientation of the alkyl residues of both compounds. Consequently, **Ib2** and **IIb2** were identified as 7-(3'-monomethylallyl-)-L-tryptophan and 7-(3'-pentenyl-)-L-tryptophan, respectively.

Due to low conversion and unsuccessful separation on HPLC, **Ic**, **IIc** and **IIIc** were elucidated from the mixture with **Ia**, **IIa** and **IIIa**, respectively. The aliphatic signals of the indole moiety and those of the alkyl or benzyl residue for **Ic** were overlapped by those of **Ia**. The aromatic signals of the indole moiety were distinct from those of **Ia**, and could be used to identify the alkylation position. Comparison with the signals of C5-alkylated[1] L-tryptophan confirmed **Ic** as C5-monomethylallyl-L-tryptophan. For **IIc** and **IIIc** comparable results were obtained and these compounds were assigned to C5-(2-pentenyl-)-L-tryptophan and C5-benzyl-L-tryptophan, respectively.

Determination of the kinetic parameters

Enzyme assays for determination of the kinetic parameters of DMAPP and its analogues MAPP, 2-pentenyl-PP and benzyl-PP contained 1 mM L-tryptophan, 5 mM $CaCl_2$ or $MgCl_2$, 0.15% (v/v) glycerol, 50 mM Tris-HCl (pH 7.5) and the respective alkyl or benzyl diphosphate in final concentrations of up to 0.5 or 1 mM. The reaction mixtures were all incubated at 37 °C. For 6-$DMATS_{Sa}$ a protein amount of 5 µg and an incubation time of 30 min were used in presence of 2-pentenyl-PP. For incubation with MAPP or benzyl-PP the protein amount and incubation time were 10µg and 60 min. The recombinant protein 6-$DMATS_{Sv}$ was assayed in amounts of 1 µg with DMAPP for 5 min and of 10 µg with other DMAPP analogues for 60 min. The assays for TyrPT contained 15 µg protein and were incubated for 60 min with 2-pentenyl-PP and 90 min with MAPP or benzyl-PP. 10 µg 5-$DMATS_{Sc}$ and an incubation time of 60 min were used for 2-pentenyl-PP. For MAPP, they were 20 µg and 90 min. In the reactions with benzyl-PP, 25 µg protein and an incubation time of 90 min were used. Kinetic parameters of 5-DMATS were obtained from enzyme assays with 20 µg of purified protein and incubation time of 60 min. The reactions were terminated with 100 µL MeOH and the protein was removed by centrifugation at 17.000 x g for 15 min.

Parameters of Michaelis-Menten kinetics such as $K_{M,}$ the turnover number k_{cat} and the catalytic efficiency k_{cat}/K_M were determined by Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots (Table S3)

Reference List

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9. Liebhold, M.; Xie, X.; Li, S.-M. *Org. Lett.* **2013,** *15*, 3062-3065.
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Table S1. Enzyme activities of several prenyltransferases towards L-tryptophan in presence of DMAPP and its analogues MAPP, 2-pentenyl-PP and benzyl-PP.

	DMAPP [%]	2-pentenyl-PP [%]	MAPP [%]	benzyl-PP [%]
6-DMATS$_{Sa}$	100	91.2	51.4	13.7
6-DMATS$_{Sv}$	100	89.7	37.8	8
TyrPT	67.9	37.8	17.5	7.8
5-DMATS$_{Sc}$	83.1	65.1	20.7	6.7
5-DMATS	100	91.9	50.9	28.5

Table S2. MS data of the enzyme products of the different prenyltransferases with L-tryptophan and DMAPP analogues.

				ESI-MS data		HR-EI/ESI-MS data		
Prenyl-transferase	*Compound*	*Alkylation/benzyl-ation position*	*Chemical formula*	*calculated*	*measured*	*calculated*	*measured*	*Deviation [ppm]*
6-DMATS$_{Sa}$	**Ia**	C-6	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	259.10 $[M+H]^+$	-	-	-
	IIa	C-6	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	273.20 $[M+H]^+$	-	-	-
	IIIa	C-6	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	295.20 $[M+H]^+$	-	-	-
6-DMATS$_{Sv}$	**Ia**	C-6	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	259.1 $[M+H]^+$	258.1368 $[M]^+$	258.1322 $[M]^+$	17.8
	IIa	C-6	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	545.4 $[2*M]^+$	272.1525 $[M]^+$	272.1557 $[M]^+$	-11.8
	IIIa	C-6	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	295.20 $[M+Na]^+$	-	-	-
TyrPT	**Ia**	C-6	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	281.12 $[M+Na]^+$	281.1266 $[M+Na]^+$	281.1258 $[M+Na]^+$	2.8
	Ib1	C-7	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	281.03 $[M+Na]^+$	281.1266 $[M+Na]^+$	281.1288 $[M+Na]^+$	-7.8
	Ib2	C-7 rev	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	281.30 $[M+Na]^+$	281.1266 $[M+Na]^+$	281.1255 $[M+Na]^+$	3.9
	IIa +IIc	C-6+C-5	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	295.18 $[M+Na]^+$	295.1422 $[M+Na]^+$	295.1433 $[M+Na]^+$	-3.7
	IIb1	C-7	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	295.10 $[M+Na]^+$	295.1422 $[M+Na]^+$	295.1395 $[M+Na]^+$	9.1
	IIb2	C-7rev	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	295.30 $[M+Na]^+$	295.1422 $[M+Na]^+$	295.1437 $[M+Na]^+$	-5.1
	IIIa+IIIc	C-6+C-5	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	317.00 $[M+Na]^+$	-	-	-
	IIIb1	C-7	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	316.90 $[M+Na]^+$	-	-	-
5-DMATS$_{Sc}$	**Ia+Ic**	C-6+C-5	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	281.04 $[M+Na]^+$	-	-	-
	Ib1	C-7	$C_{15}H_{18}N_2O_2$	258.32 $[M]^+$	281.10 $[M+Na]^+$	-	-	-
	IIa+IIc	C-6+C-5	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	295.20 $[M+Na]^+$	295.1422 $[M+Na]^+$	295.1399 $[M+Na]^+$	7.8
	IIb1	C-7	$C_{16}H_{20}N_2O_2$	272.34 $[M]^+$	295.20 $[M+Na]^+$	295.1422 $[M+Na]^+$	295.1448 $[M+Na]^+$	-8.8
	IIIa+IIIc	C-6+C-5	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	295.09 $[M+H]^+$	-	-	-
	IIIb1	C-7	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	317.20 $[M+Na]^+$	-	-	-
5-DMATS	**IIIa+IIIc**	C-6+C-5	$C_{18}H_{18}N_2O_2$	294.35 $[M]^+$	295.14 $[M+Na]^+$	-	-	-

- not meassured

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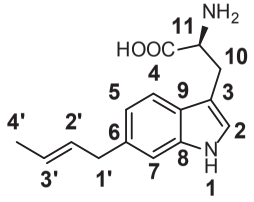
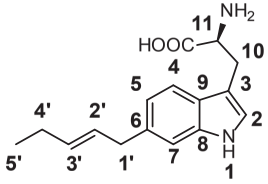
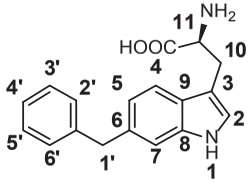
Table S3. Kinetic parameters of the tested prenyltransferases towards DMAPP and analogues thereof (MAPP, 2-pentenyl-PP and benzyl-PP).

	DMAPP			**2-pentenyl-PP**			**MAPP**			**benzyl-PP**		
	K_M [mM]	k_{cat} [s^{-1}]	k_{cat} / K_M [s^{-1} M^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat} / K_M [$s^{-1}M^{-1}$]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
6-DMATS$_{Sa}$	0.095	0.63	6630	0.011	0.0031	280	0.025	0.0011	44	0.036	0.0012	33
6-DMATS$_{Sv}$	0.025	0.17	6570	0.030	0.0039	130	0.038	0.0011	29	0.098	0.00089	9.1
5-DMATS$_{Sc}$	0.05[a]	0.4[a]	8000[a]	0.030	0.0014	46.7	0.057	0.00051	8.9	0.105	0.00029	2.8
TyrPT	0.39[b]	0.0036[b]	9.2[b]	0.031	0.00089	2.9	0.025	0.0011	44	0.082	0.00048	5.9
5-DMATS	0.076[c]	1.3[c]	17105[c]	0.131[d]	0.023[d]	174[d]	0.04[d]	0.005[d]	136[d]	0.116	0.0015	12.9

[a,b,c,d] Data from previous publications (Ozaki,T *et al.*, 2013[7]; Fan, A. *et. al.*, 2014[10];Yu,X. *et al.*, 2012;Liebhold *et al.*, 2013[4]; Liebhold and Li,2012[1])

Table S4. ^{1}H-NMR data of C6-alkylated L-tryptophan

Comp	6-monomethylallyl-L-tryptophan (**Ia**)	6-(2-pentenyl-)-L-tryptophan (**IIa**)	6-benzyl-L-tryptophan (**IIIa**)
			
Pos.	$\delta_{H,}$ *multi,* J	$\delta_{H,}$ *multi,* J	$\delta_{H,}$ *multi,* J
2	7.12, s	7.13, s	7.13, s [a]
4	7.60, dd, 8.1, 0.4	7.60, dd, 8.2, 0.4	7.60, dd, 8.2, 0.6
5	6.89, dd, 8.1, 1.4	6.90, dd, 8.2, 1.4	6.93, dd, 8.2, 1.5
6	-		
7	7.15, d, 0.7	7.16, d, 0.7	7.18, s [b]
10	3.49, ddd, 15.2, 4.0, 0.6	3.49, dd, 15.2, 4.0	3.48, dd, 15.1, 4.1, 0.7
	3.11, dd, 15.2, 9.5	3.12, dd, 15.2, 9.5	3.11, dd, 15.1, 9.4
11	3.84, dd, 9.5, 4.0	3.84, dd, 9.5, 4.0	3.82, dd, 9.4, 4.1
1′	3.37, d, 6.7	3.38, d, 6.0	4.04, s
2′	5.60, dtq, 15.1, 6.6, 1.4	5.50–5.63, m	7.17–7.25, m [b]
3′	5.51, dqt, 15.1, 6.3, 1.3	5.50–5.63, m	7.17–7.25, m [b]
4′	1.67, ddt, 6.2, 1.4, 1.3	2.04, m	7.11–7.15, m [a]
5′		0.99, t, 7.5	7.17–7.25, m [b]
6′			7.17–7.25, m [b]
solvent	3.31 (CD_3OD)	3.31 (CD_3OD)	3.31 (CD_3OD)

[a,b] signals with same letters are overlapping each other

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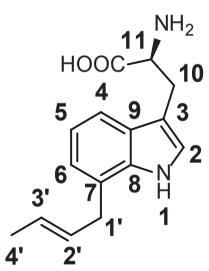
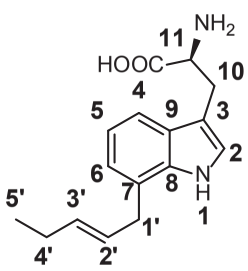
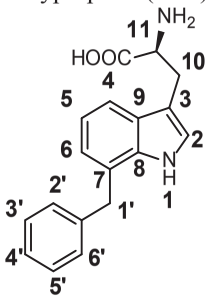
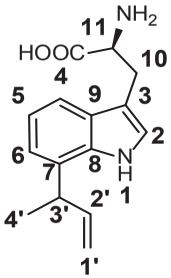
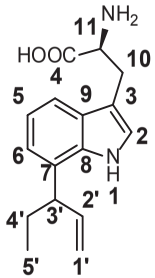
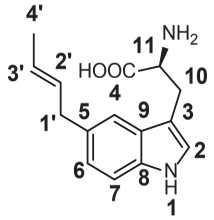
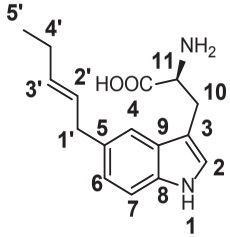
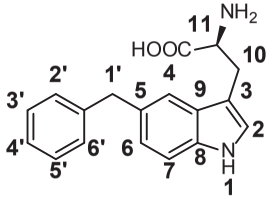
Table S5. [1]H-NMR data of C7-alkylated L-tryptophan

Comp	7-monomethylallyl-L-tryptophan (**Ib1**) 	7-(2-pentenyl-)-L-tryptophan (**IIb1**) 		7-benzyl-L-tryptophan (**IIIb**) 	7-(3'-monomethylallyl-)-L-tryptophan (**Ib2**) 	7-(3'-pentenyl-)-L-tryptophan (**IIb2**) 
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_C	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
2	7.19, s	7.19, s	124.7	7.18, s	7.20, s	7.16, s
3			109.9			
4	7.56, dd, 7.9, 1.0	7.55, dd, 7.8	117.1	7.58, dd, 8.1, 0.8	7.56, dd, 7.8, 1.1	7.55, dd, 7.8, 1.1
5	7.00, dd, 7.9, 7.1	7.00, t, 7.5	120.2	7.01, t, 7.6	7.03, t, 7.6	7.01, t, 7.5
6	6.93, dd, 7.1, 0.6	6.93, dd, 7.0	122.2	6.92, dd, 7.2, 0.4	6.98, d, 7.1	6.96, d, 7.4
7	-	-	125.1	-	-	-
8			136.8			
9			128.4			
10	3.51, ddd, 15,4, 4.0, 0.9[c] 3.14, dd, 15.4, 9.5	3.50, ddd, 15.1, 3.8 3.13, dd, 15.1, 9.3	28.4	3.50, dd, 15.1. 4.0 3.13, dd, 15.1, 9.4	3.51, ddd, 15.1, 4.1, 0.9 3.15, dd, 15.1, 9.3	3.41 [§] 3.04, dd, 14.8, 8.7
11	3.85, dd, 9.5, 4.0	3.84, dd, 9.3, 3.8	56.5	3.83, dd, 9.4, 4.0	3.85, m, 9.3, 4.1	3.71 [§]
1´	3.53, d, 5.2 [c]	3.54, d, 3.3	35.0	4.20, s	5.12, dt, 17.3, 1.7 5.04, dt, 10.3, 1.7	5.08, dt, 17.2, 1.5 4.99, ddd, 10.2, 1.9, 1.0
2´	5.65, dtq, 15.1, 6.4, 1.4	5.58–5.67, m	127.9	7.20–7.26, m	6.12, ddd, 17.3, 10.3, 6.3	6.06, ddd, 17.2, 10.2, 7.6
3´	5.57, dqt, 15.1, 6.2, 1.2	5.58–5.67, m	134.1	7.20–7.26, m	3.89, m	3.59 [§]
4´	1.66, ddt, 6.0, 1.4, 1.3	2.03, m	26.1	7.14, m	1.44, d, 7.0	1.86, m
5´		0.97, t, 7.5	13.8	7.20–7.26, m		0.89, t, 7.4
6´				7.20–7.26, m		
solvent	3.31 (CD_3OD)	3.31 (CD_3OD)		3.31 (CD_3OD)	3.31 (CD_3OD)	3.31 (CD_3OD)

[c] signals with same letters are overlapping each other; [§] coupling constants not determinable, due to low signal intensity

Table S6. ^{1}H-NMR data of C5-alkylated L-tryptophan

Comp	5-monomethylallyl-L-tryptophan (**Ic**)	5-(2-pentenyl-)-L-tryptophan (**IIc**)	5-benzyl-L-tryptophan (**IIIc**)
			
Pos.	δ_H, *multi*, *J*	δ_H, *multi*, *J*	δ_H, *multi*, *J*
2	7.15 *	7.15 #	7.16, s
4	7.50, s	7.50, s	7.60, dd, 1.5, 0.7
5	-	-	-
6	6.96, dd, 8.5, 1.7	6.97, dd, 8.3, 1.6	6.97, dd, 8.3, 1.6
7	7.27, dd, 8.4, 0.6	7.27, d, 8.2	7.27, dd, 8.3, 0.5
10	3.51 *	3.48 #	3.51 $
	3.09 *	3.09 #	3.09 $
11	3.84 *	3.83 #	3.84 $
1′	3.40 *	3.40 #	4.05, s
2′	5.61 *	5.50–5.66 #	7.17–7.25 $
3′	5.51 *	5.50–5.66 #	7.17–7.25 $
4′	1.67 *	2.04 #	7.13 $
5′		0.99 #	7.17–7.25 $
6′			7.17–7.25 $
solvent	3.31 (CD_3OD)	3.31 (CD_3OD)	3.31 (CD_3OD)

*,#,$ signals with same symbols are overlaying with those of the corresponding C6-alkylated L-tryptophan derivatives (**Ia** – **IIIa**)

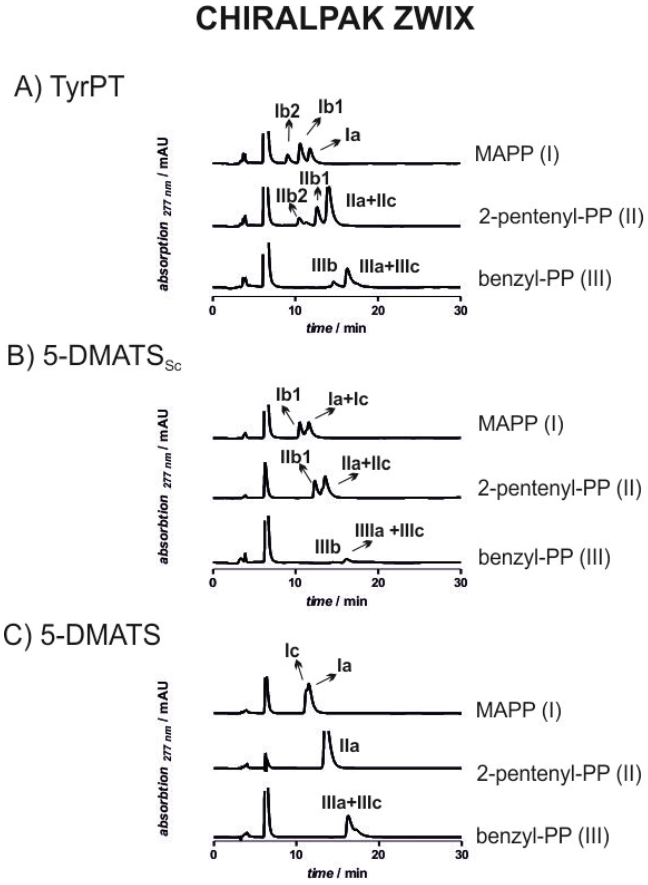


Figure S1. HPLC analysis of the reaction mixtures of L-tryptophan with MAPP (**I**), 2-pentenyl-PP (**II**) and benzyl-PP (**III**) on a Chiralpak Zwix (+) column. The enzyme assays of 100 µL contained 1 mM L-tryptophan, 2 mM alkyl diphosphate, 5 mM $CaCl_2$ and 7.5 µM of purified protein were incubated at 37°C for 16 h.

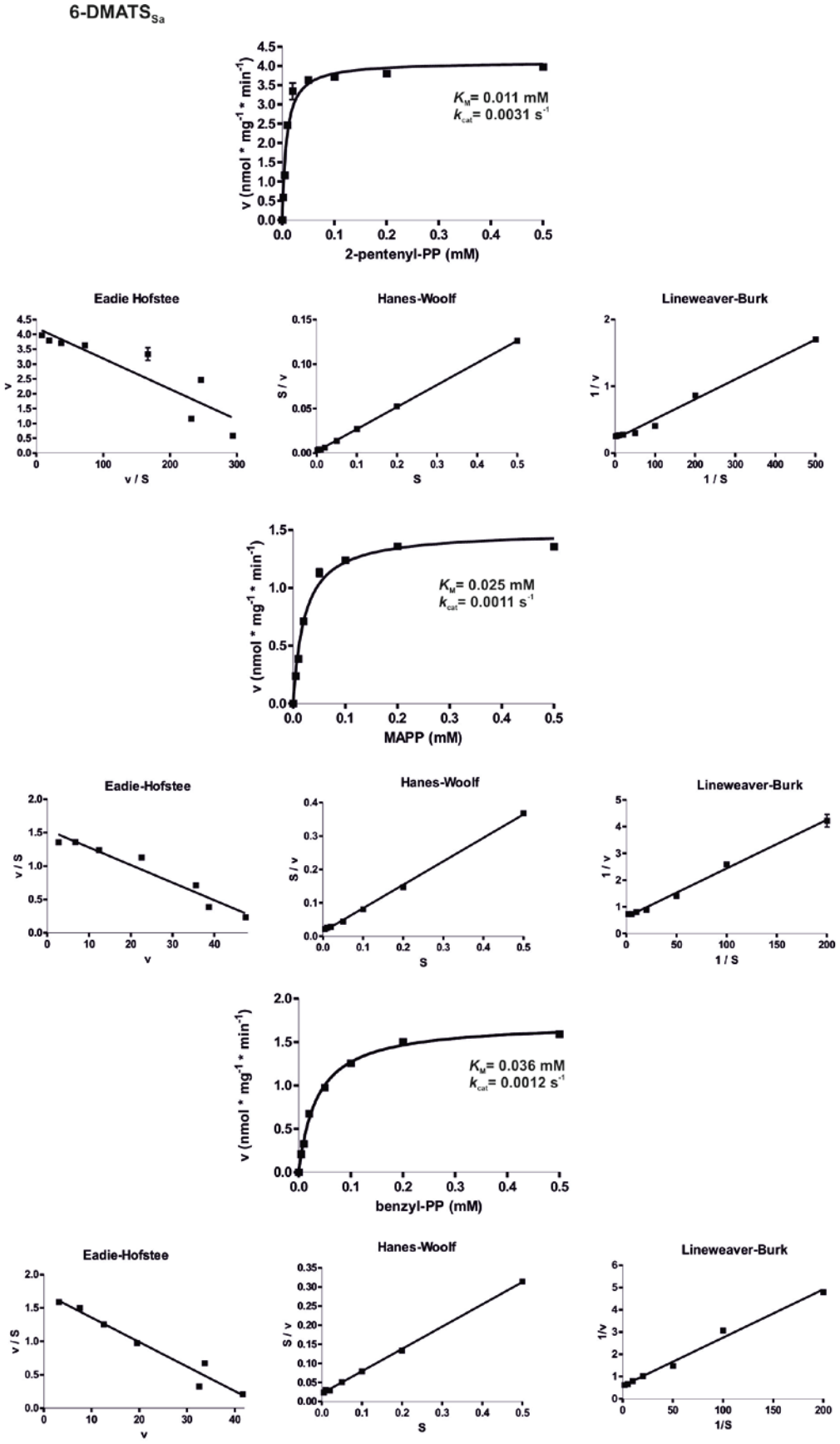


Figure S2. Dependence of the product formation of the 6-DMATS$_{Sa}$ reaction on 2-pentenyl-PP, monomethylallyl-PP (MAPP) or benzyl-PP in the presence of L-tryptophan.

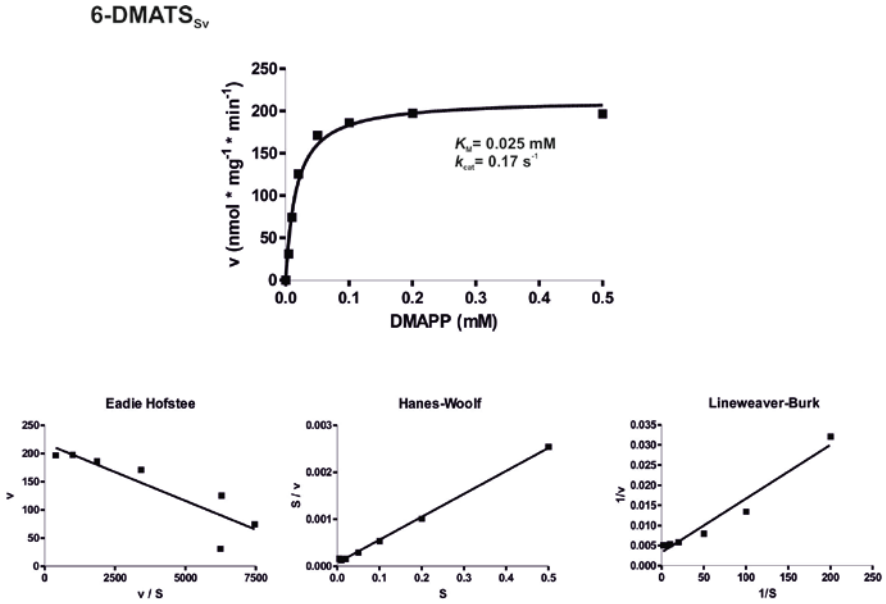


Figure S3. Dependence of the product formation of the 6-DMATS$_{Sv}$ reaction on DMAPP in the presence of L-tryptophan.

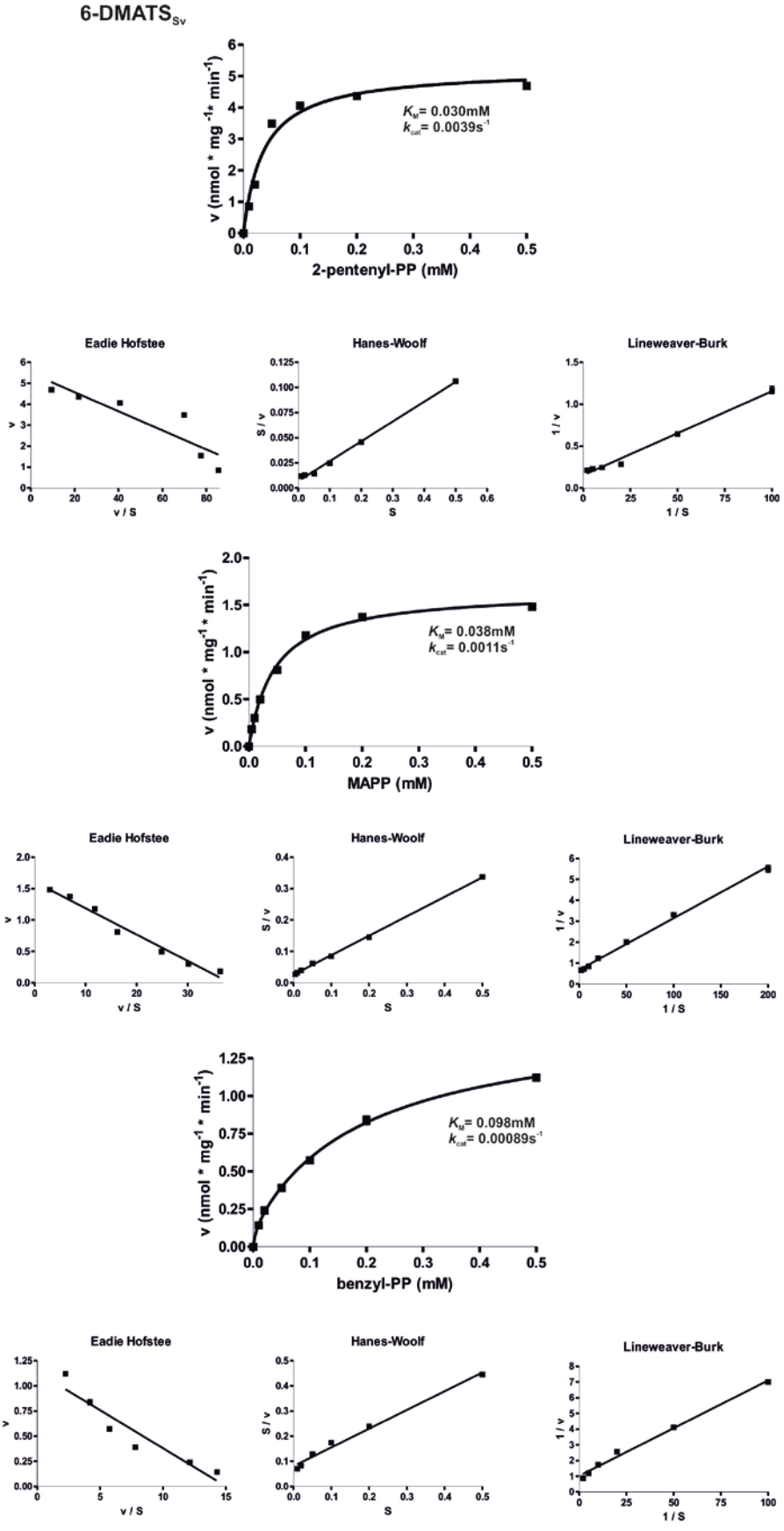


Figure S4. Dependence of the product formation of the 6-DMATS$_{Sv}$ reaction on 2-pentenyl-PP, monomethylallyl-PP (MAPP) or benzyl-PP in the presence of L-tryptophan.

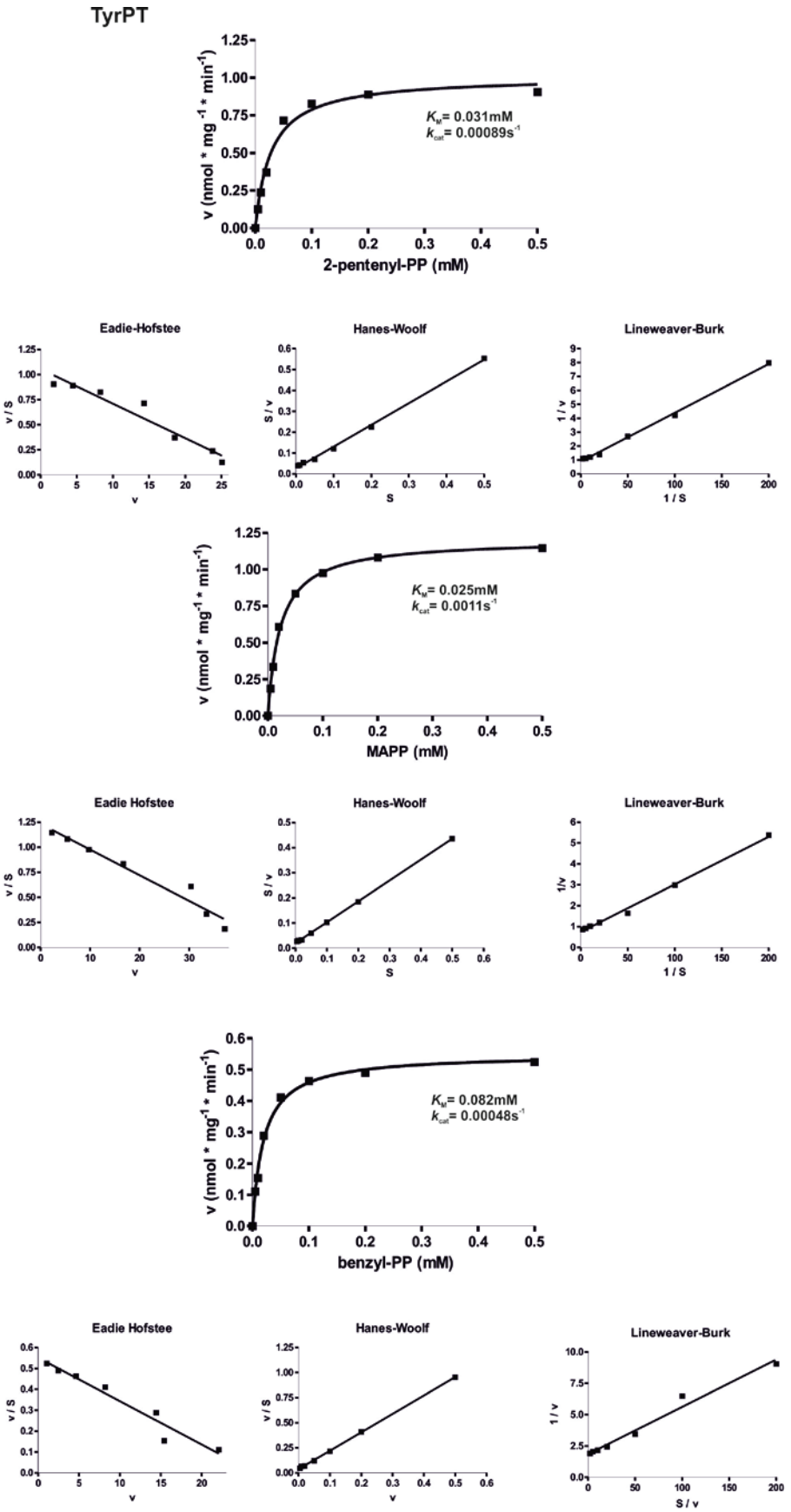


Figure S5. Dependence of the product formation of the TyrPT reaction on 2-pentenyl-PP, monomethylallyl-PP (MAPP) or benzyl-PP in the presence of L-tryptophan.

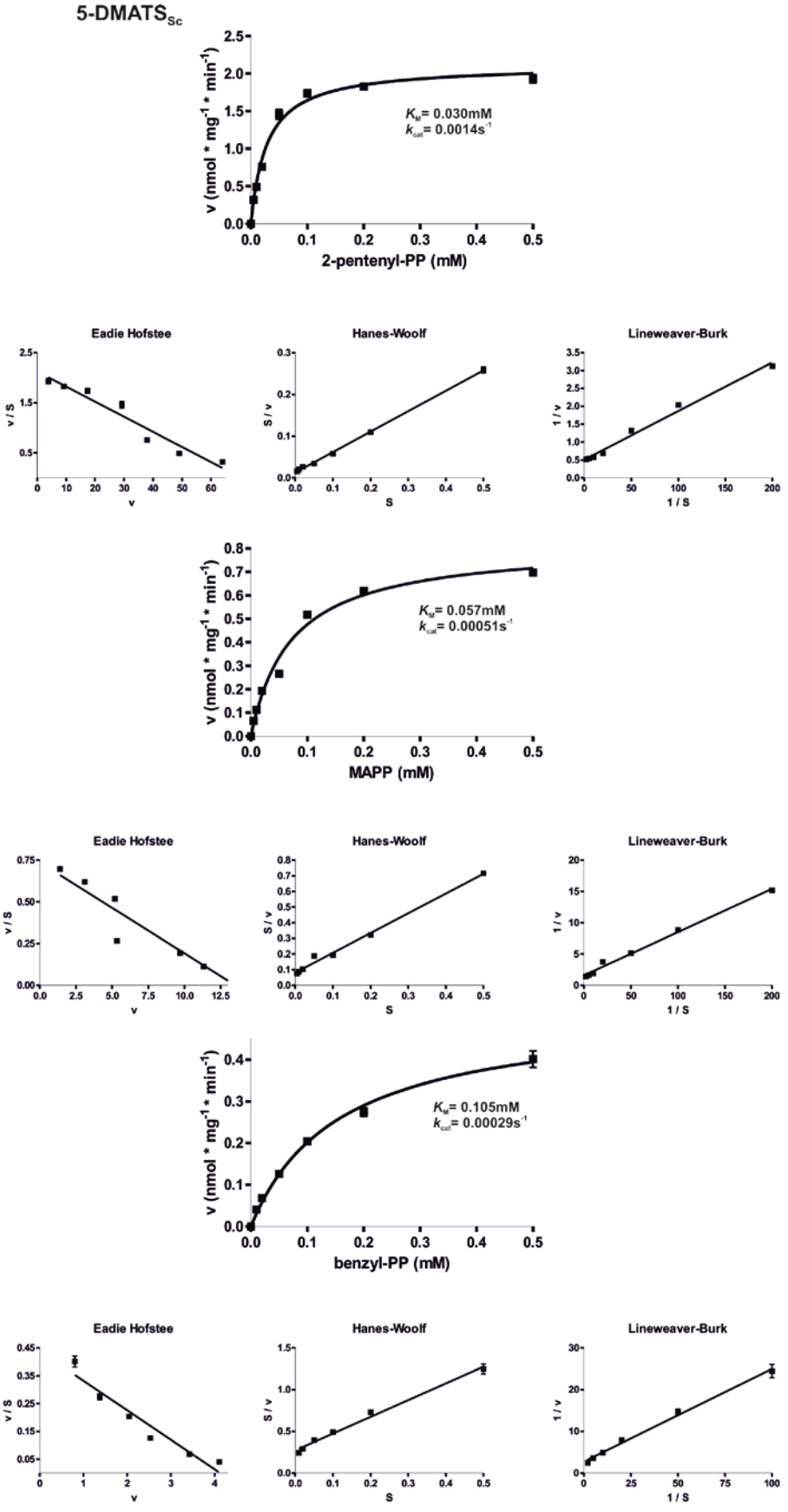


Figure S6. Dependence of the product formation of the 5-DMATS$_{Sc}$ reaction on 2-pentenyl-PP, monomethylallyl-PP (MAPP) or benzyl-PP in the presence of L-tryptophan.

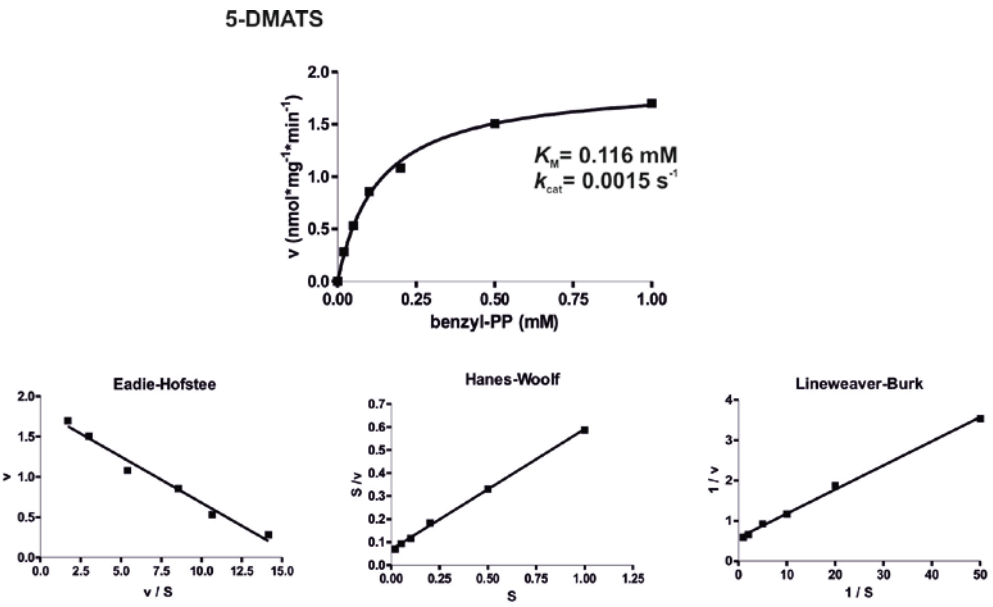


Figure S7. Dependence of the product formation of the 5-DMATS reaction on benzyl-PP in the presence of L-tryptophan.

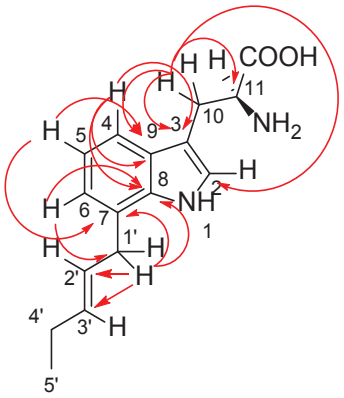


Figure S8. HMBC connectivities of 7-(2-pentenyl-)-L-tryptophan (**IIb1**).

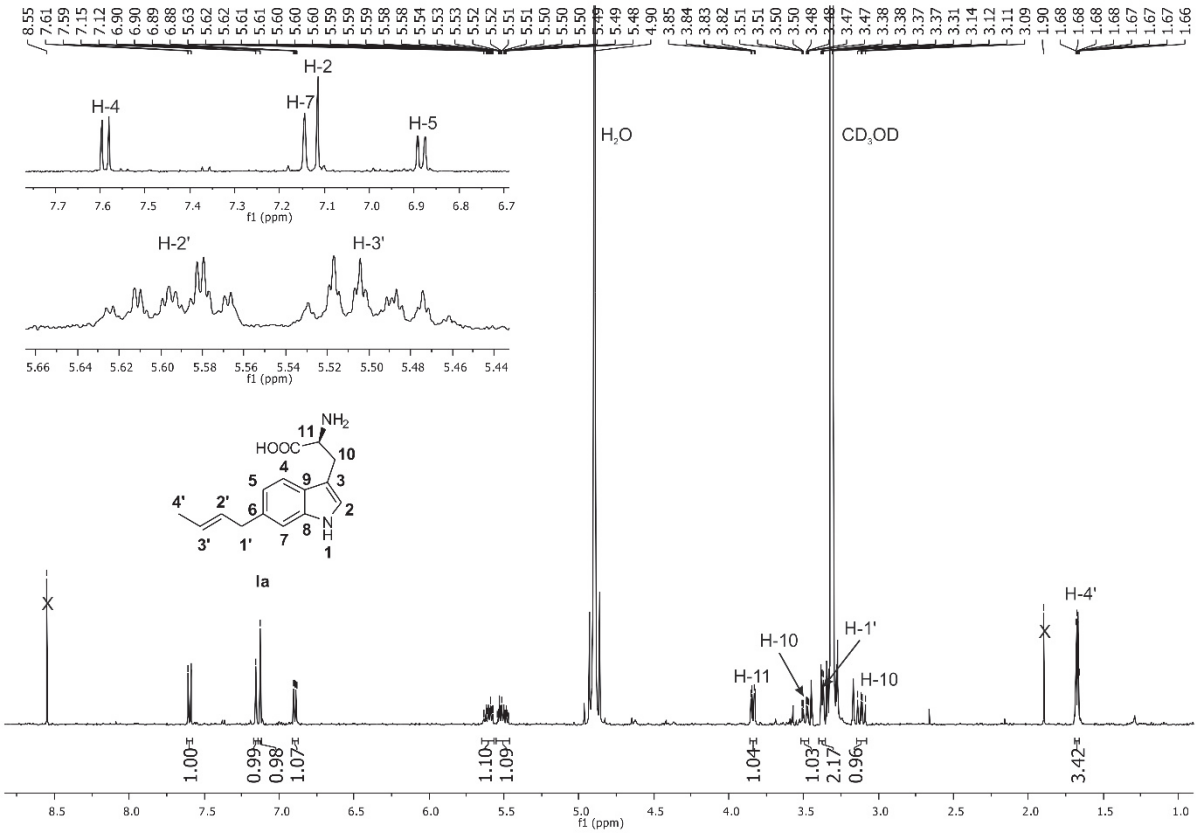


Figure S9. ^{1}H-NMR spectrum of 6-monomethylallyl-L-tryptophan (**Ia**) in CD_3OD.

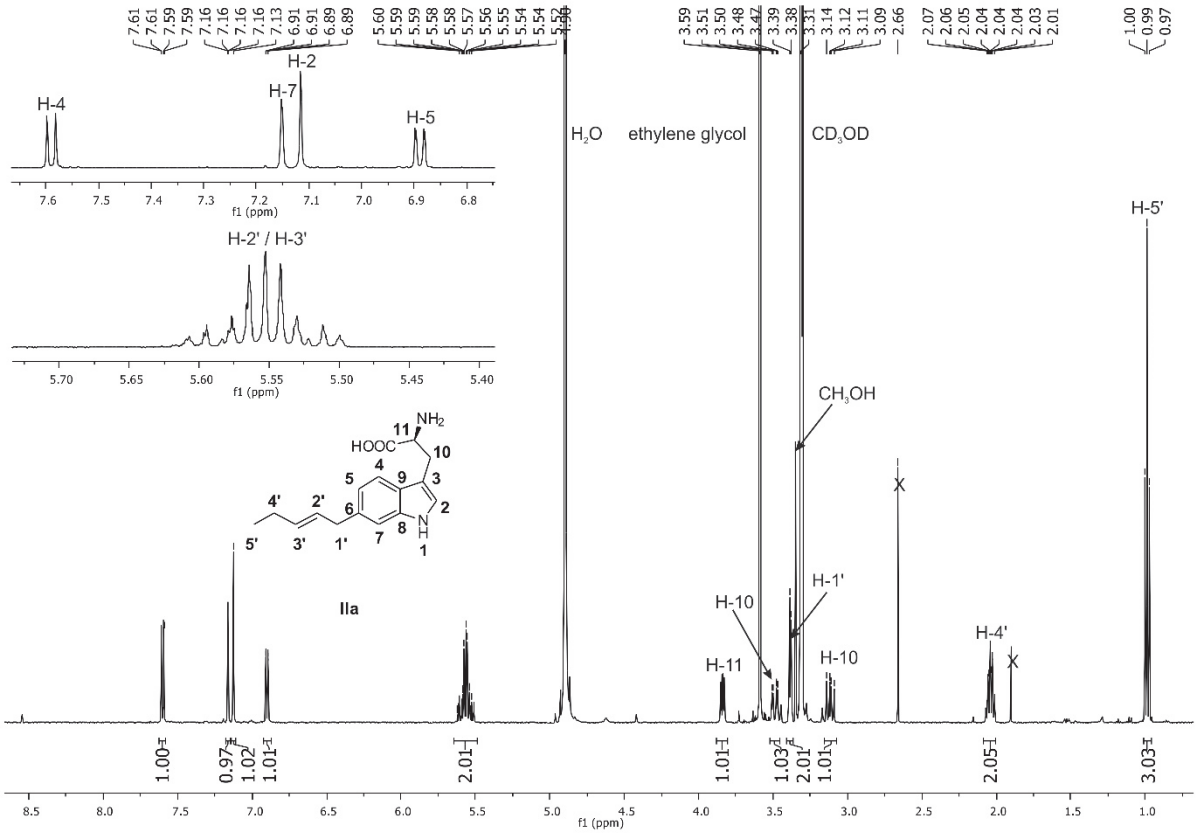


Figure S10. ^{1}H-NMR spectrum of 6-(2-pentenyl-)-L-tryptophan (**IIa**) in CD_3OD.

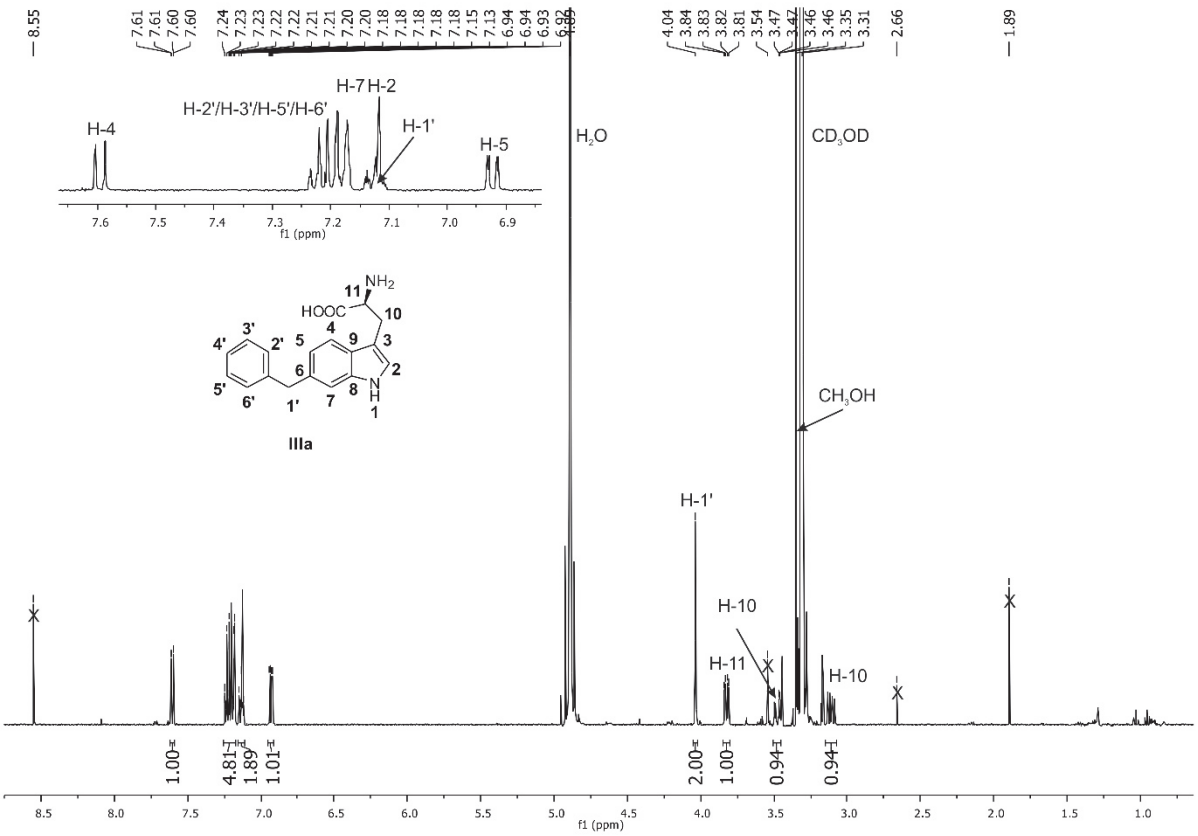


Figure S11. ^{1}H-NMR spectrum of 6-benzyl-L-tryptophan (**IIIa**) in CD_3OD.

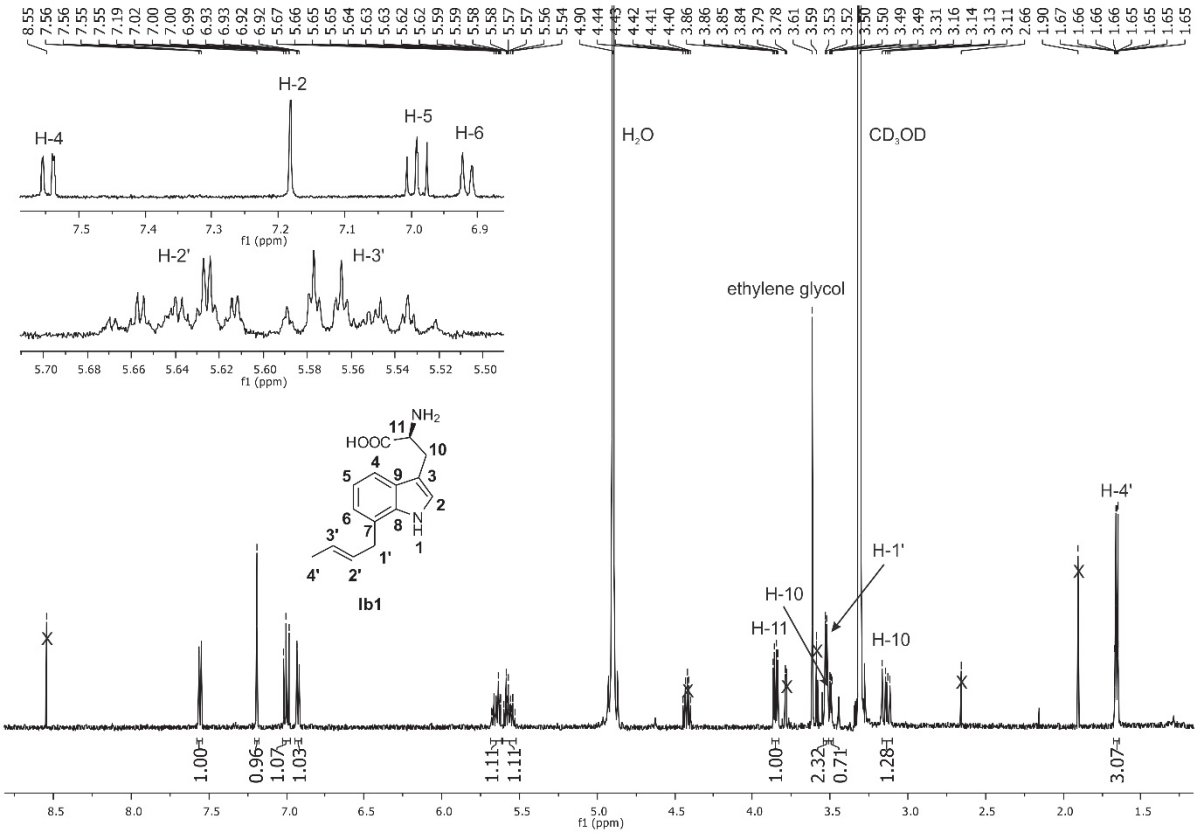


Figure S12. ^{1}H-NMR spectrum of 7-monomethylallyl-L-tryptophan (**Ib1**) in CD_3OD.

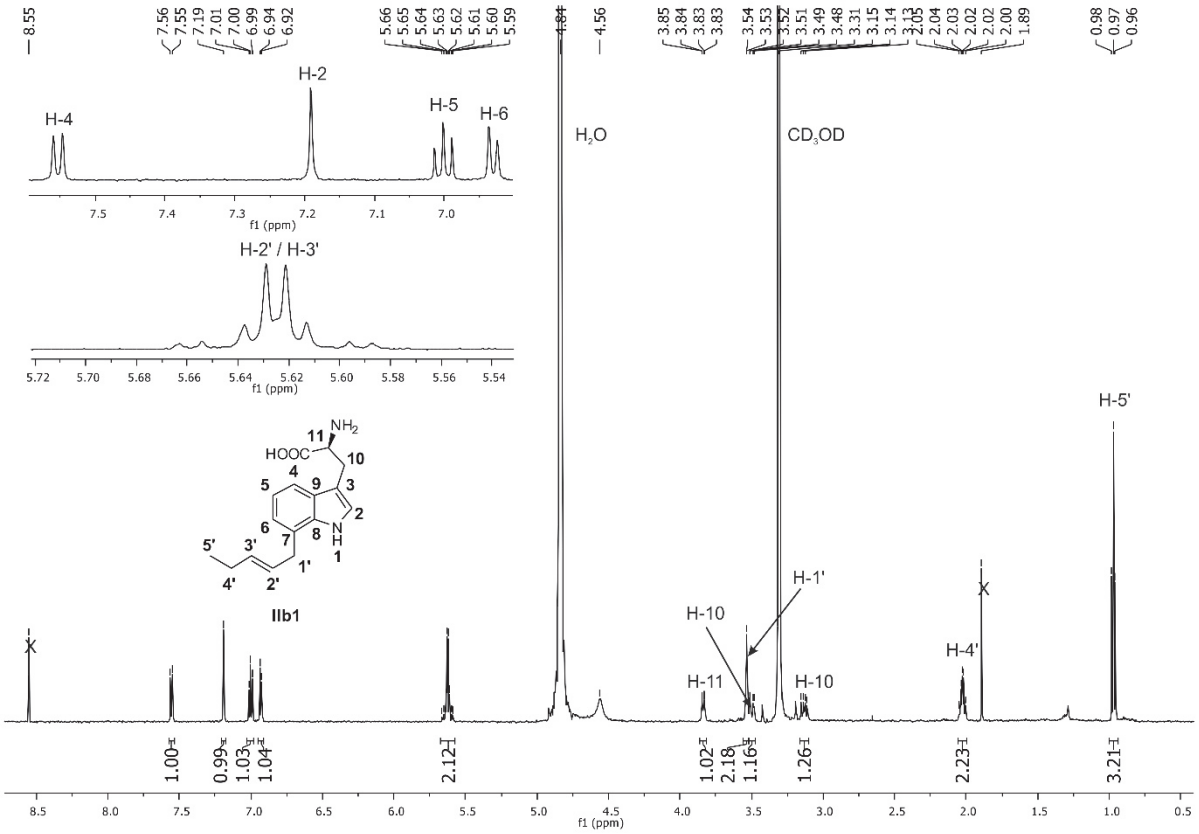


Figure S13. ^{1}H-NMR spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

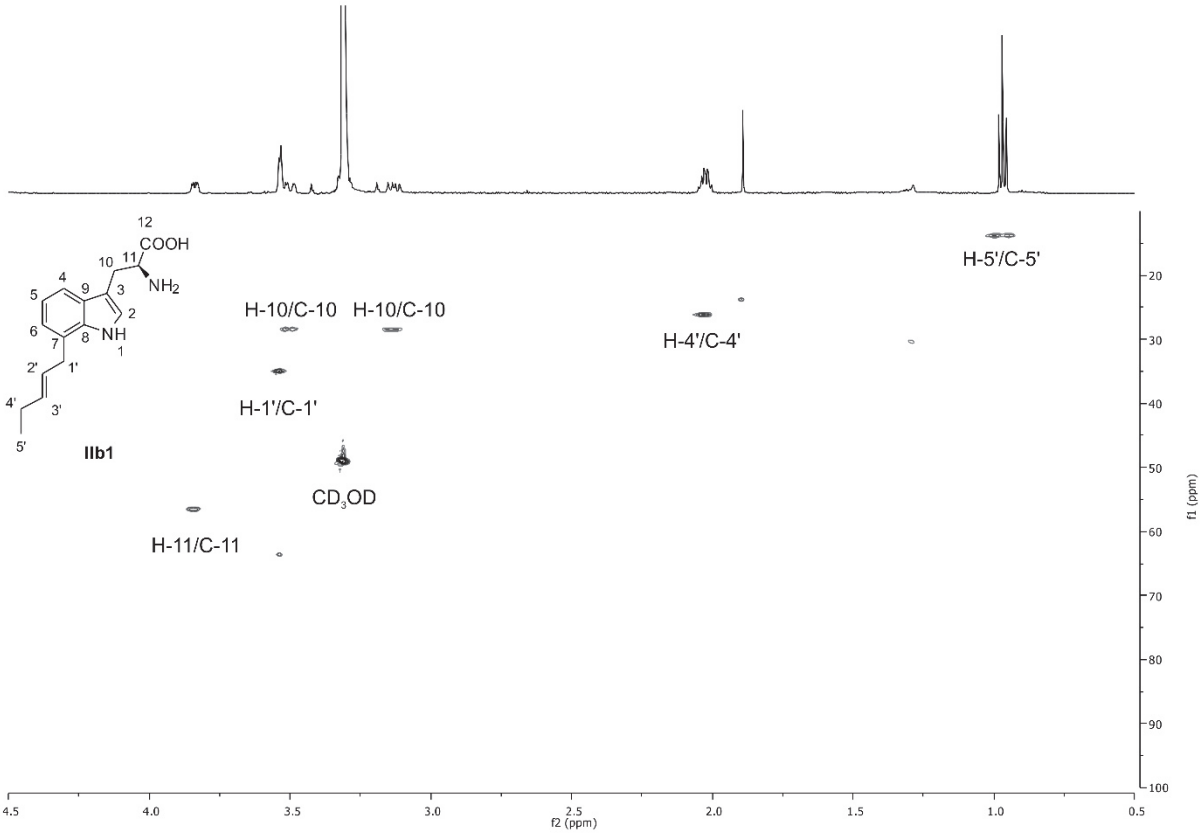


Figure S14. HSQC spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

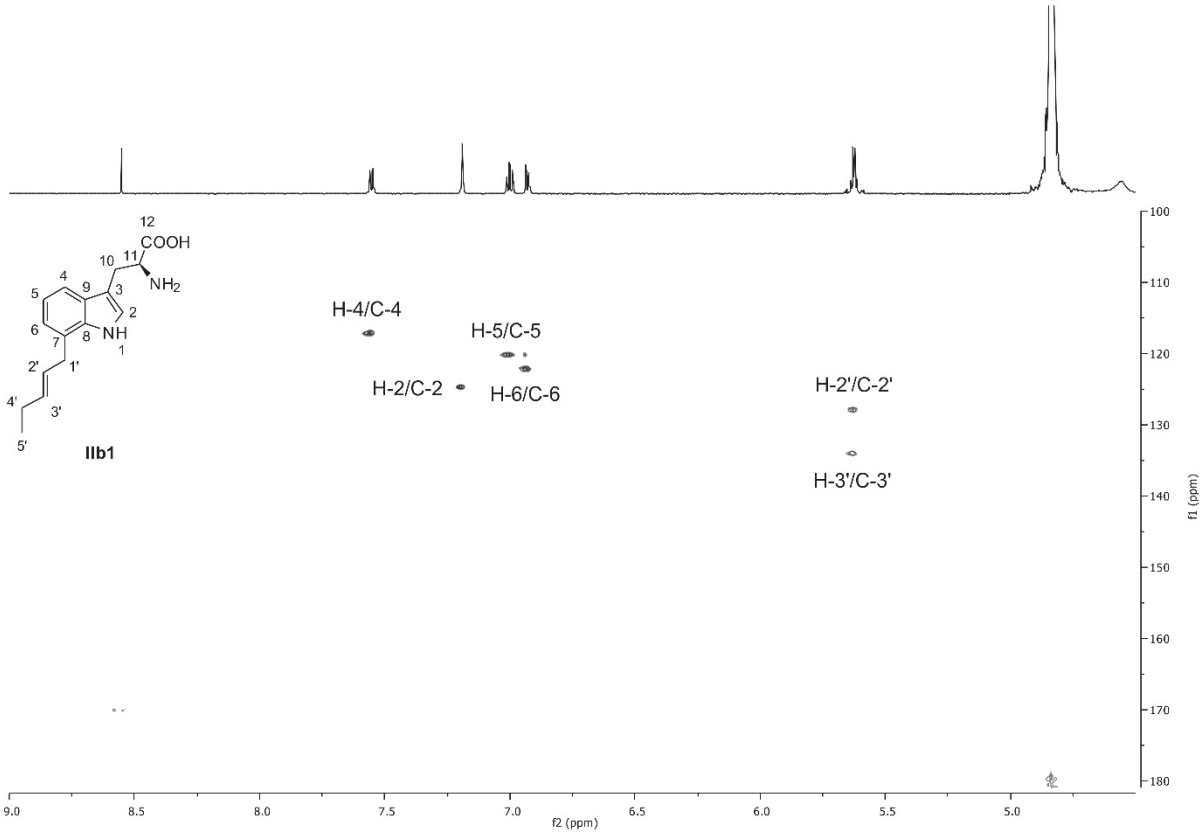


Figure S15. HSQC spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

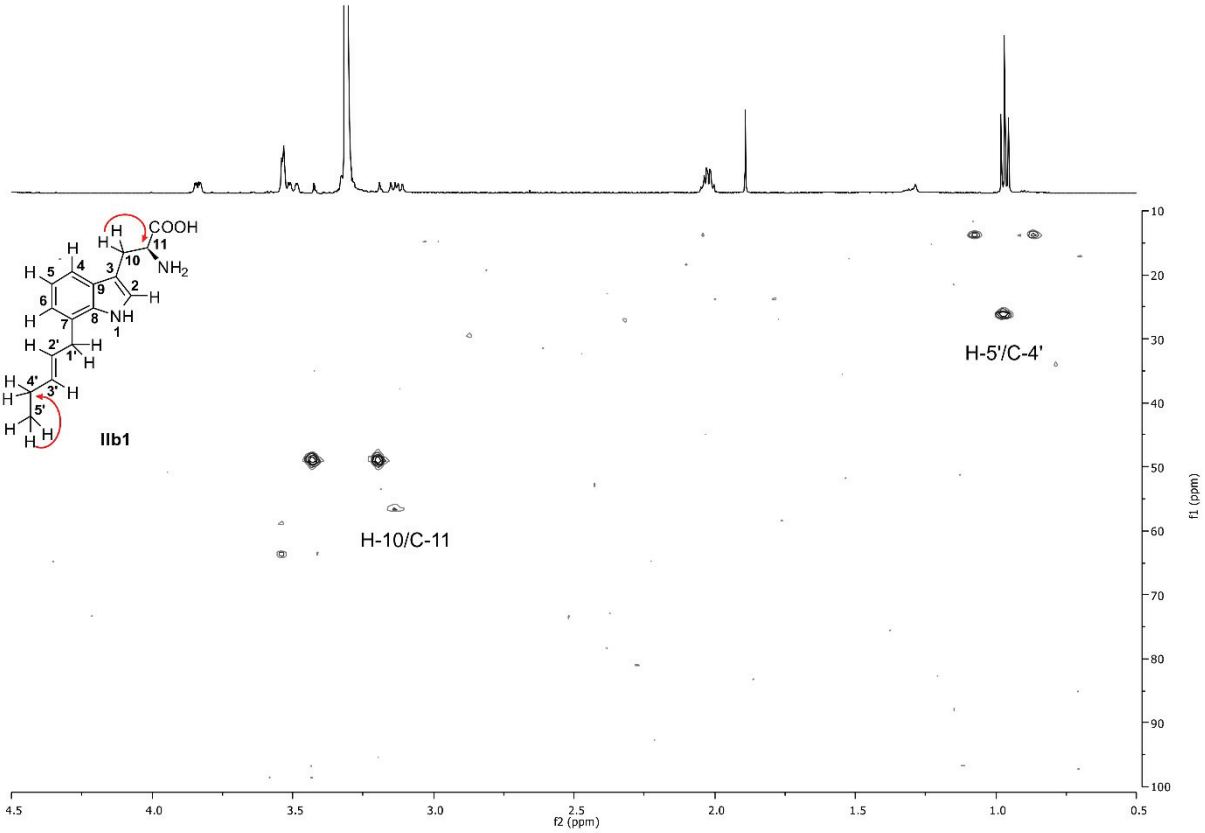


Figure S16. HMBC spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

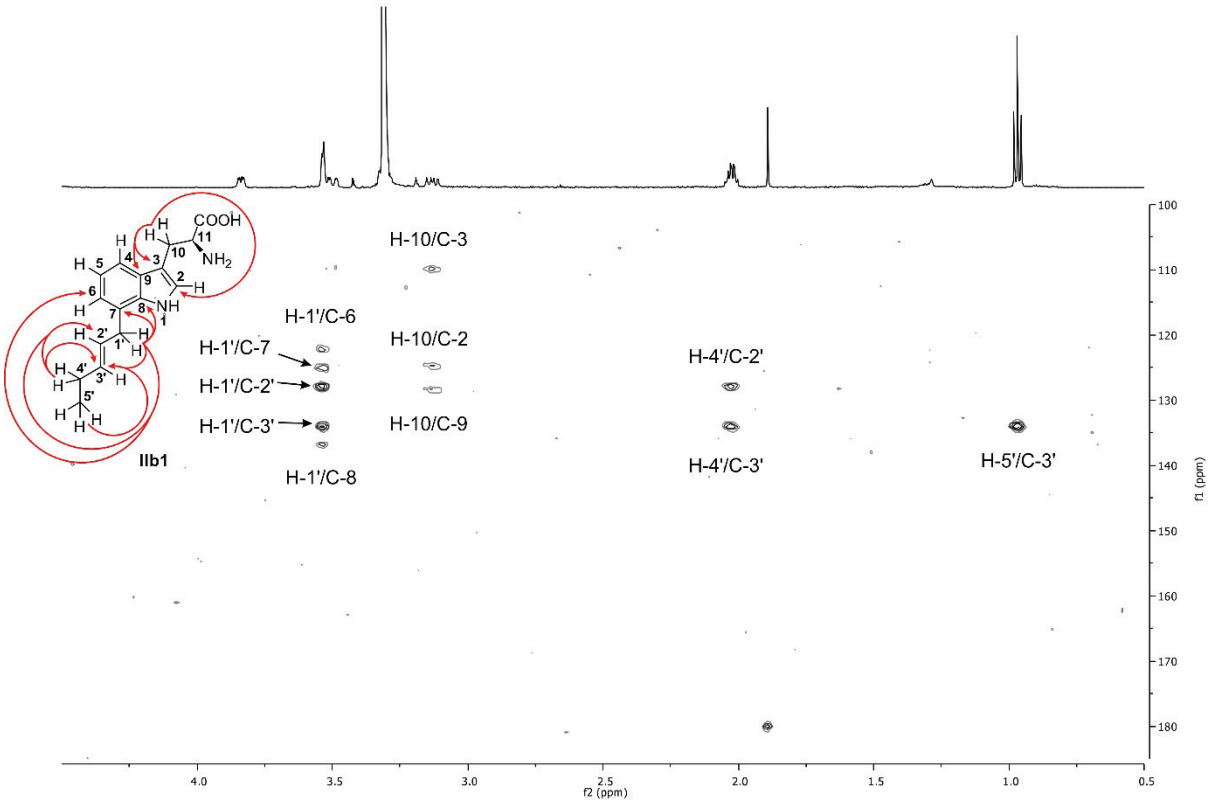


Figure S17. HMBC spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

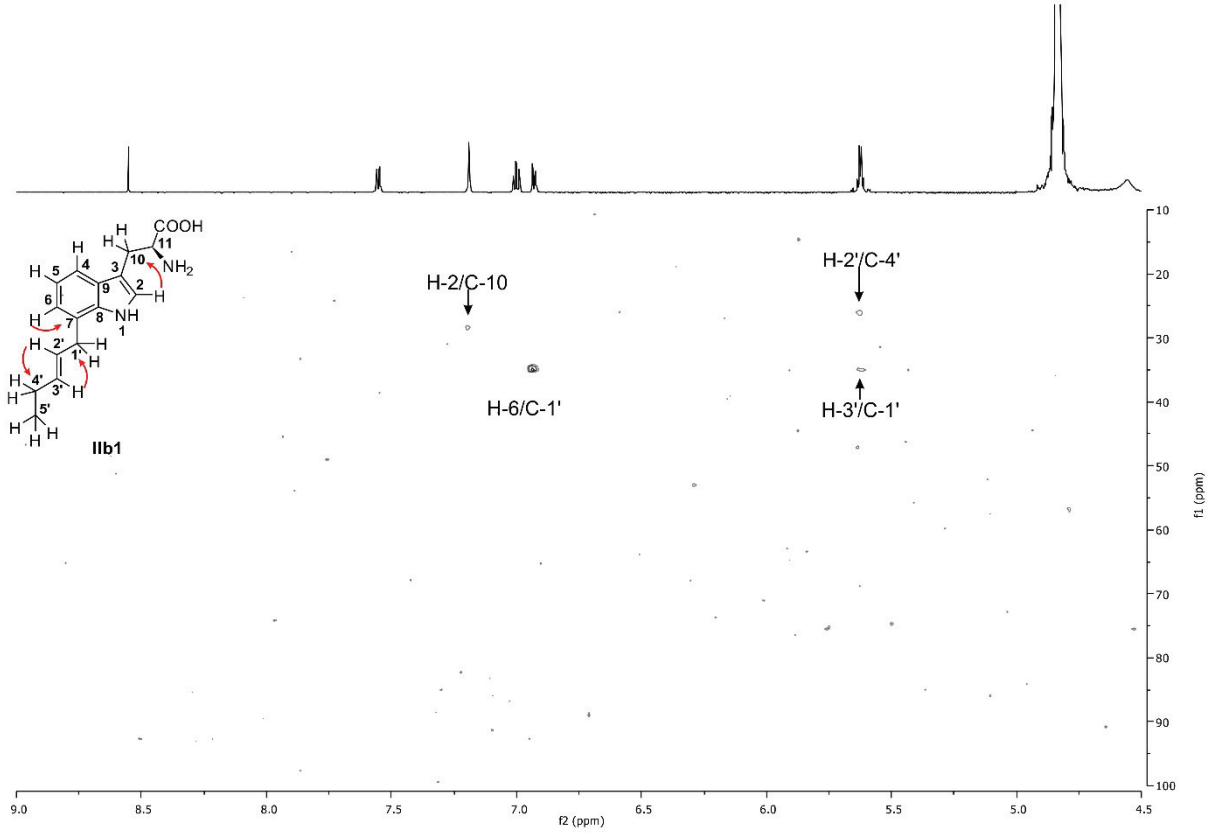


Figure S18. HMBC spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

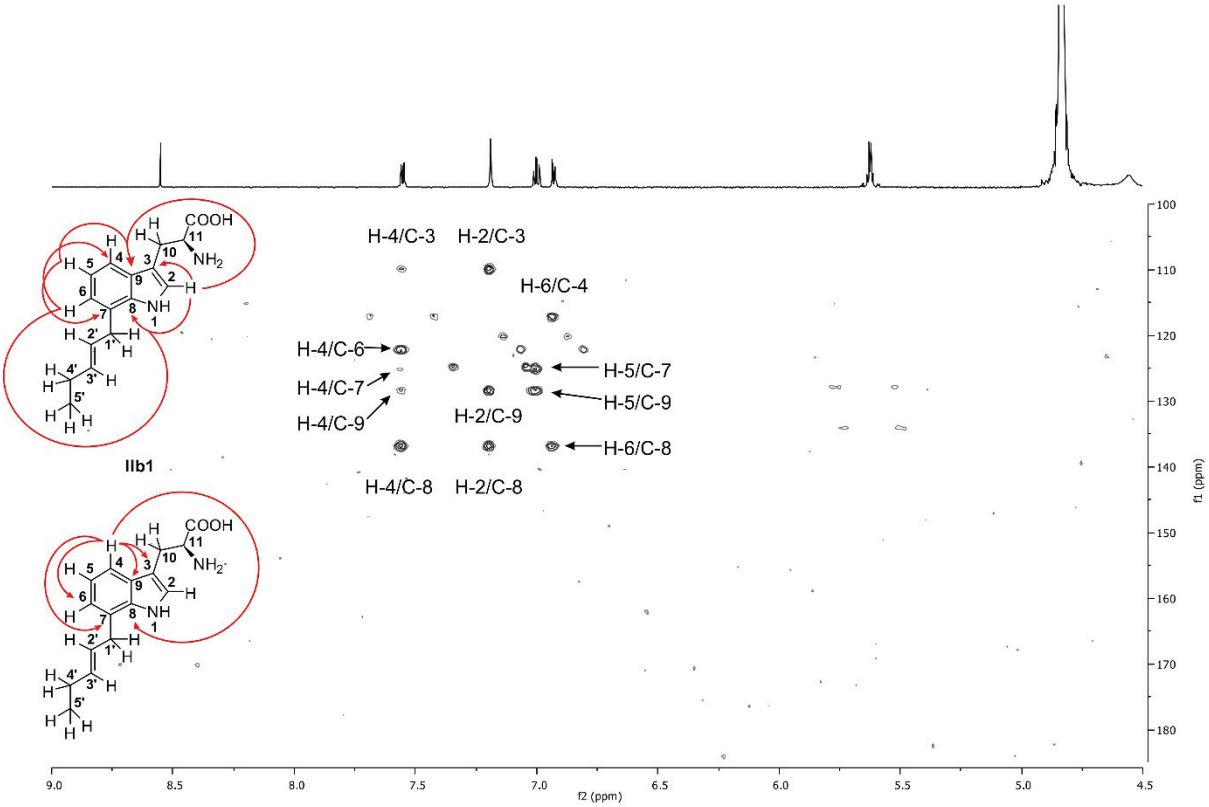


Figure S19. HMBC spectrum of 7-(2-pentenyl-)-L-tryptophan (**IIb1**) in CD_3OD.

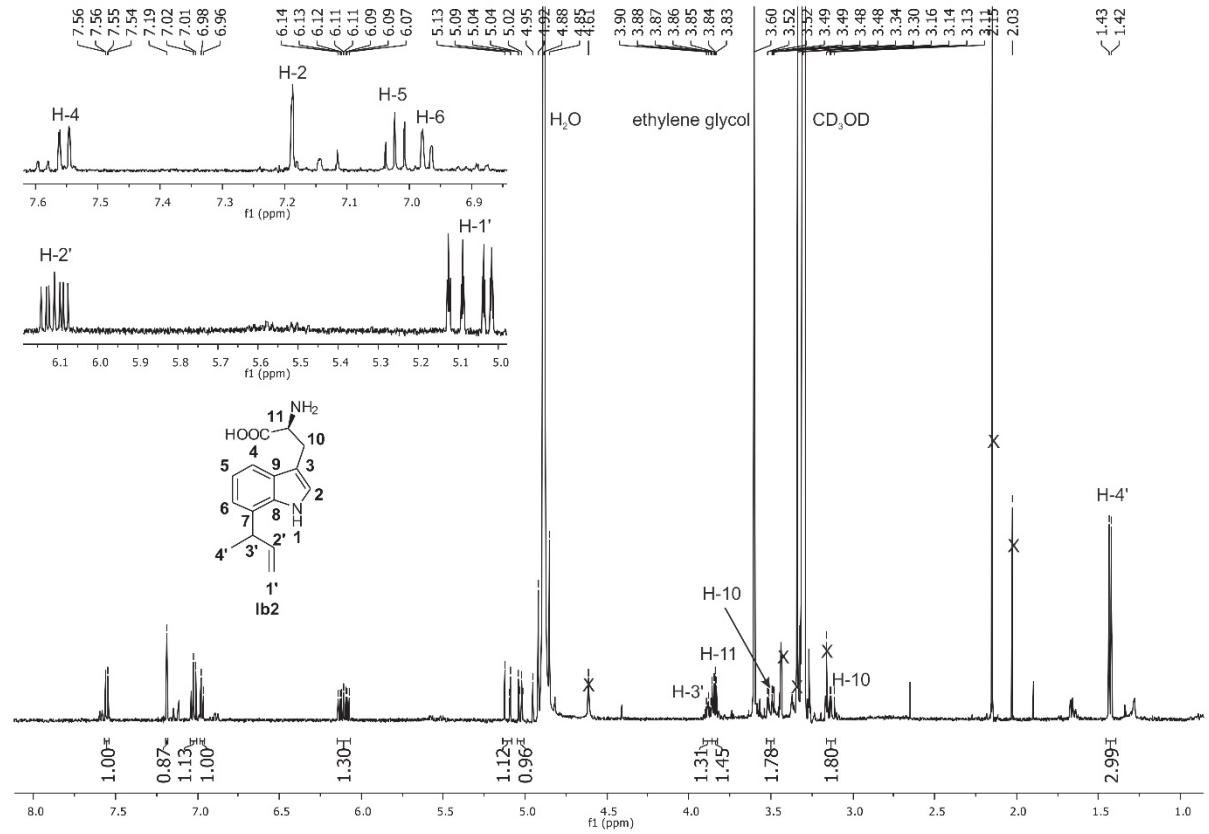


Figure S20. ^{1}H-NMR spectrum of 7-(3'-monomethylallyl-)-L-tryptophan (**Ib2**) in CD_3OD.

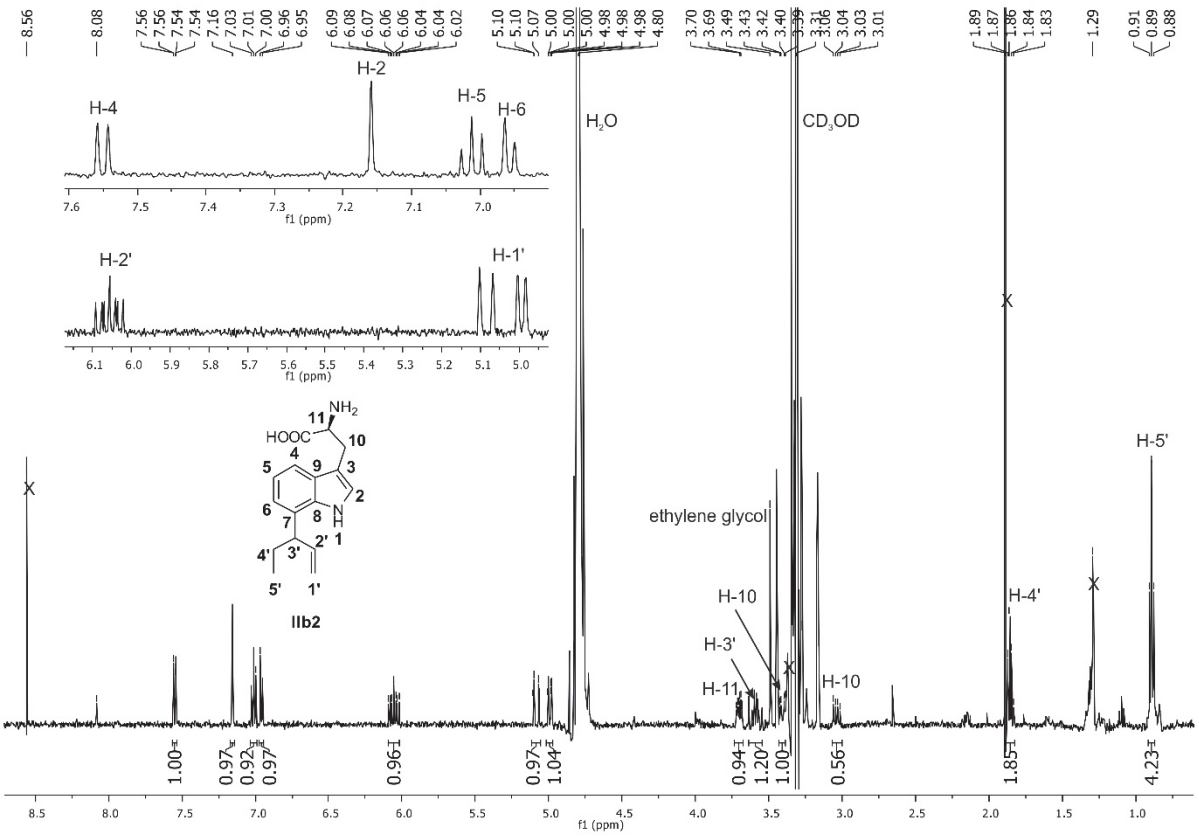


Figure S21. ^{1}H-NMR spectrum of 7-(3'-pentenyl-)-L-tryptophan (**IIb2**) in CD_3OD.

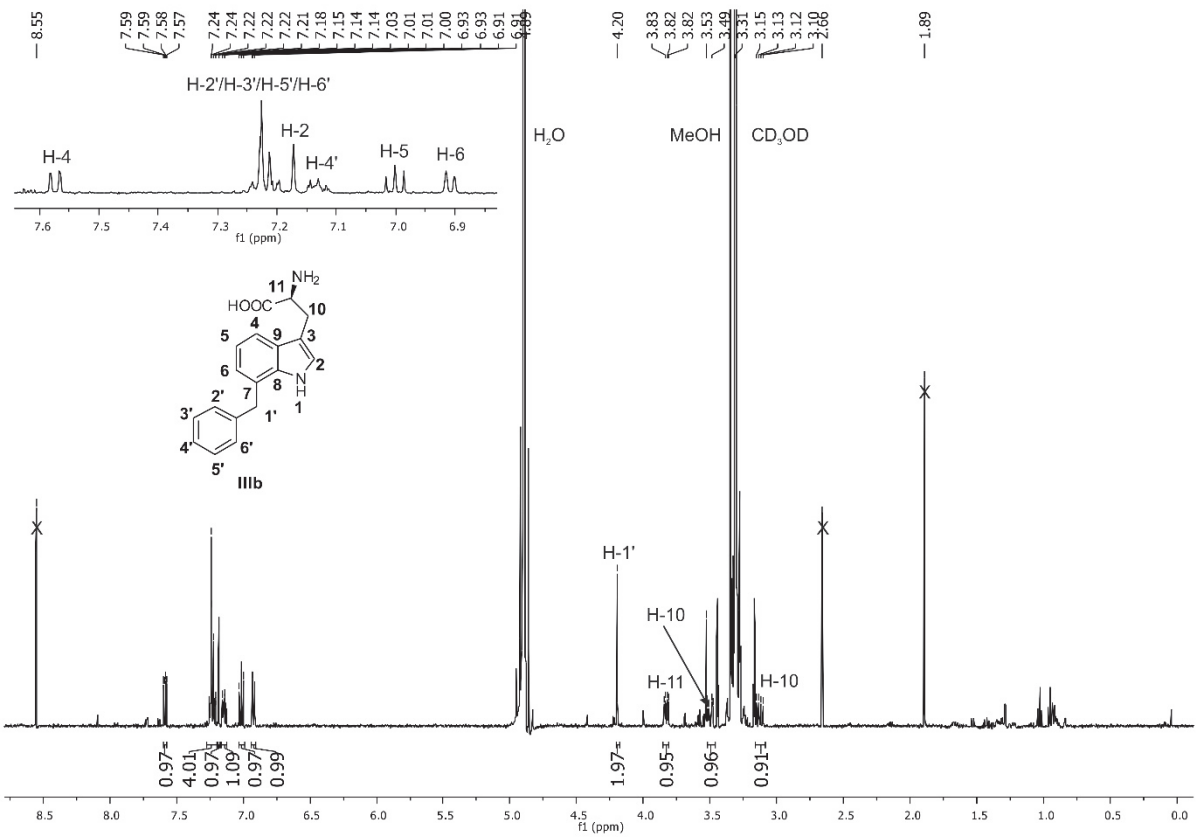


Figure S22. ^{1}H-NMR spectrum of 7-benzyl-L-tryptophan (**IIIb**) in CD_3OD.

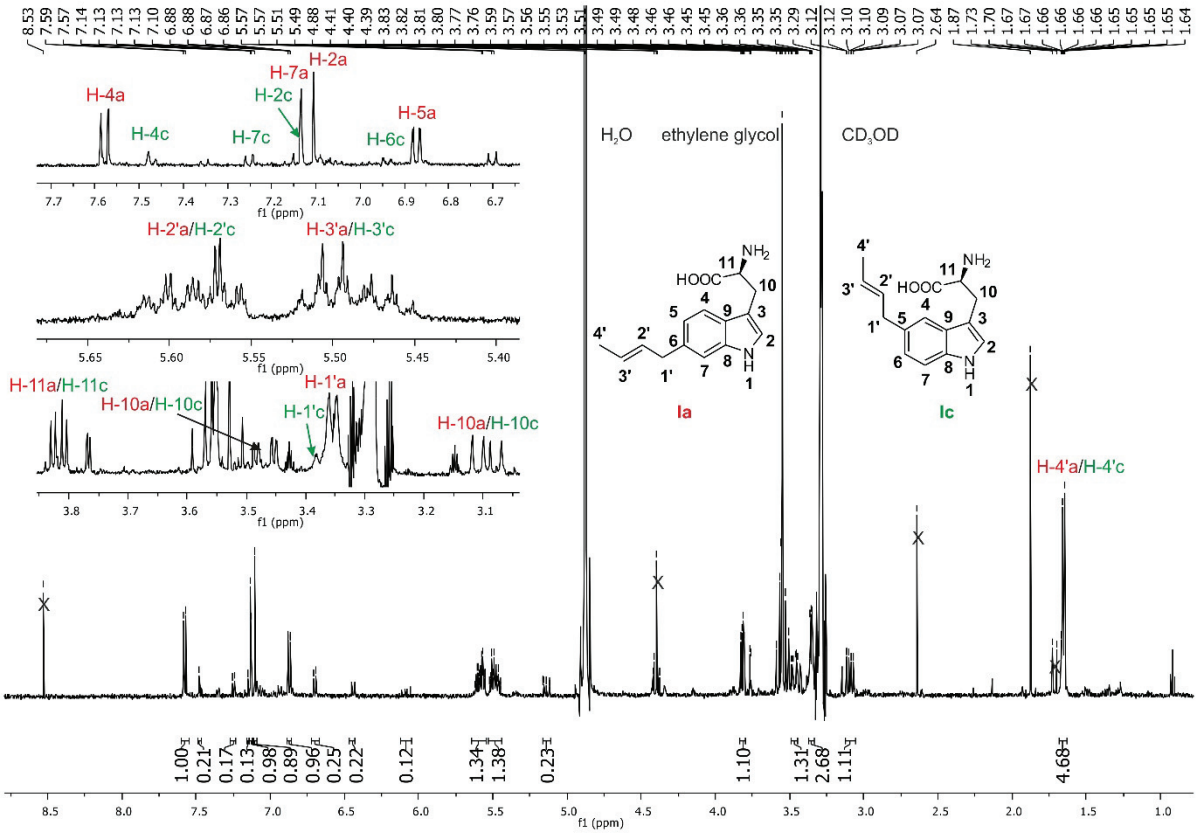


Figure S23. ^{1}H-NMR spectrum of 6-monomethylallyl-L-tryptophan (**Ia**) and 5-monomethylallyl-L-tryptophan (**Ic**) in CD_3OD.

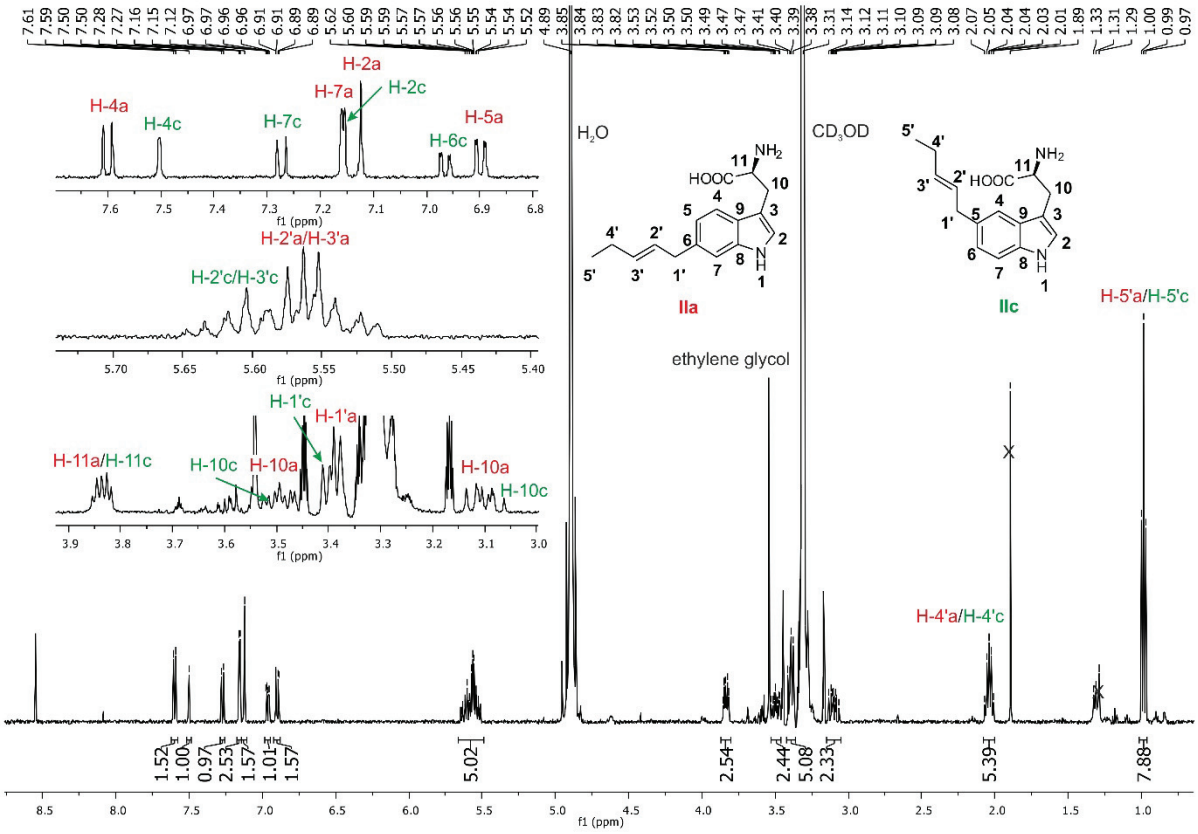


Figure S24. ^{1}H-NMR spectrum of 6-(2-pentenyl-)-L-tryptophan (**IIa**) and 5-(2-pentenyl-)-L-tryptophan (**IIc**) in CD_3OD.

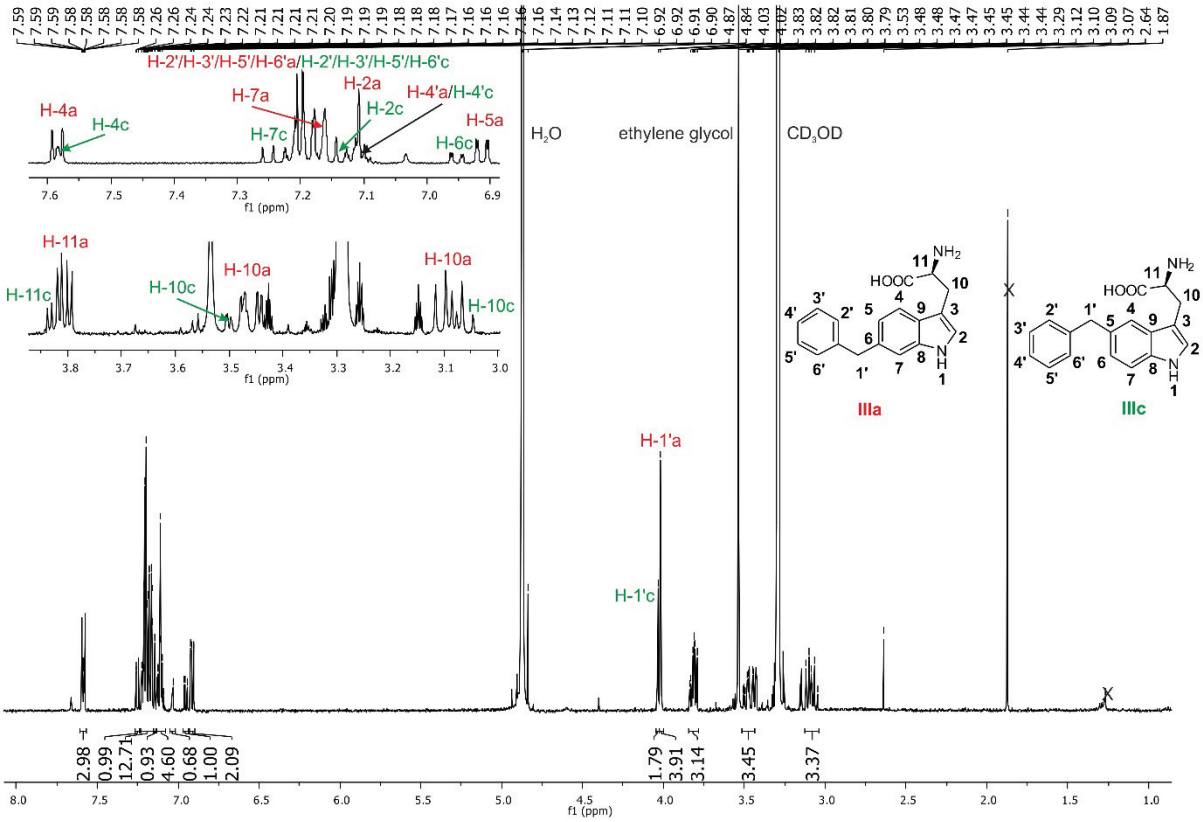


Figure S25. ^{1}H-NMR spectrum of 6-benzyl-L-tryptophan (**IIIa**) and 5-benzyl-L-tryptophan (**IIIc**) in CD_3OD.

5. Conclusions and future prospects

In this thesis, novel strategies for chemoenzymatic synthesis of alkylated compounds were developed by using prenyltransferases of the DMATS superfamily and unnatural DMAPP analogues.

Initially, four alkyl diphosphates were synthesized and their reactions with three L-tryptophan prenyltransferases investigated. The obtained results proved that the existence of the double bond in DMAPP and its localization at β-position are essential for the catalytic transfer reactions. The regioselectivity of the prenyltransferases was reduced and the alkylation position was shifted partially or completely. Five unnaturally regular alkylated L-tryptophan products were obtained from the reaction with two prenyltransferases. Incubation of five dipeptide prenyltransferases with the respective natural or best-accepted substrates and two DMAPP analogues provided further insights into the catalytic properties of enzymes of the DMATS superfamily. The formation of C2- as well as C3-reverse alkylated compounds showed the complete loss of the regioselectivity of these enzymes. Due to the new stereo center formed during the reverse alkylations, the enzymes catalyzed the formation of respective diastereomers. The enzymatic reactions yielded 27 reverse alkylated tryptophan-containing cyclic dipeptides. Furthermore, one new benzyl diphosphate was synthesized. FgaPT2 successfully catalyzed the regiospecific benzylation reaction of L-tryptophan and simple indole derivatives. The enzyme produced eight C5- and two C6-benzylated tryptophan derivatives. Finally, the three alkyl and benzyl donors were tested in the presence of tryptophan C5-, C6- and C7-prenylating enzymes from fungi and bacteria. Several product were formed in different ratios by these prenyltransferases, with C-6 being the favored position for the alkyl or benzyl attachment. From the enzyme assays of these five prenyltransferases, nine regular and two reverse alkylated L-tryptophan derivatives have been characterized.

The acceptance of the unnatural alkyl and benzyl donors by several prenyltransferases significantly expands the potential use of these enzymes as catalysts for Friedel–Crafts alkylations.

For future prospects, the following works should be performed:

- Investigations on the described DMAPP analogues and unnatural substrates in the presence of prenyltransferases of the DMATS superfamily to create new unnatural alkylated compounds.

- Synthesis of additional conjugated allyl DMAPP analogues and testing their acceptance by prenyltransferases.
- Synthesis of allyl and benzyl DMAPP analogues carrying functional groups and testing their acceptance by prenyltransferases.
- Mutational studies on different prenyltransferases, to enhance or alter the catalytic properties towards DMAPP analogues as well as to further understand the structural and mechanistic relationship of prenyltransferases.
- Examination of acquired or altered pharmacological properties of unnaturally alkylated indoles via cytotoxicity experiments.

6. References

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Statutory Declaration

Ich, Mike Liebhold, versichere, dass ich meine Dissertation:

„Biochemical Investigations on Microbial Prenyltransferases in the Presence of DMAPP Analogues“

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen bedient habe. Alle vollständig oder sinngemäß übernommenen Zitate sind als solche gekennzeichnet.

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Marburg, den.....

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Mike Liebhold

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Curriculum vitae

Page 201 (Curriculum vitae) contains personal data. It is therefore not part of the online-publication of this thesis.