

# Epimerization-free C-term Activation of Peptide Fragments by Ball-Milling

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# **Epimerization-free C-term Activation of Peptide Fragments by Ball-Milling**

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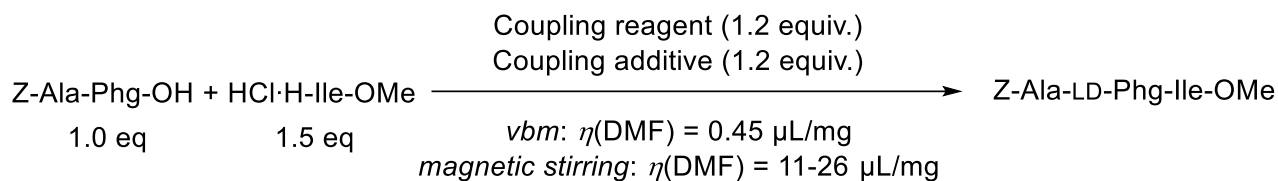
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**Abstract:** Ball-milling enabled to perform [2+1], [2+2], and [2+3] peptide couplings with high yields and, if any, very low epimerization. Very good results were obtained with peptide fragments containing highly epimerization-prone and/or highly hindered amino acids at C-term such as phenylglycine, cysteine and valine. Ball-milling was clearly identified as the key element to obtain both high yield and purity along with low epimerization. Indeed, the ball-milling conditions proved to be superior to the classical solution synthesis approach on a various array of widely used coupling agents. These results open avenues for the development of highly efficient, convergent and flexible peptide synthesis strategies based on peptide fragment couplings mediated by ball-milling.

Peptides can be found in many and diverse applications as nutriments, cosmetics and pharmaceuticals. In the field of medicinal chemistry, they can act as therapeutics and diagnostic agents, including for the fight against the Covid-19 disease.<sup>1-2</sup> Over the years, interest in this class of compounds has increased at a steady rate,<sup>3-7</sup> due to their good biological activities at very low doses, high selectivity, and high probability of success at clinical trial stages.<sup>4, 8</sup> In 2019, the FDA has estimated that approximately 100 peptide drugs were marketed in the U.S., Europe, and Japan, accounting for annual global sales ranging between \$15 billion to \$20 billion.<sup>6</sup> Among these peptide-based drugs, we can highlight Macrilen, which was discovered in our institute (IBMM) in 1999 and that was recently approved by both FDA and EMA to be used in the diagnosis of patients with adult growth hormone deficiency.<sup>9</sup> When the stepwise synthesis of low molecular weight peptides do not generally present any difficulty, the chemical synthesis of longer peptides and proteins is a much more challenging task. Indeed, the efficiency of coupling and deprotection reactions decreases with the length of the peptide chain, leading to amino acid deletions and uncompleted sequences. These difficulties have certainly slowed down, if not prevented, commercialization of some long peptide chains, and may also explain why recombinant protein strategy is generally preferred when sequences of more than 60 amino acid residues are targeted.<sup>10</sup> Significant efforts have been deployed by peptide scientists to tackle this challenge, mainly by using convergent fragment coupling approaches, in particular using native chemical ligation (NCL) of peptides fragments.<sup>11-13</sup> Yet, these approaches suffer from a limited scope: peptide bond disconnections cannot be performed at any point of the peptide chain. For instance, a N-terminal cysteine or an amino acid modified by a thiol auxiliary are required to perform NCL. Limitations are even more constraining when coupling a C-term activated peptide fragment with a free N-term fragment; the risk of epimerization can be extremely high, leading to the production of highly undesirable diastereomers.<sup>14</sup> This hurdle can be overcome: some examples are reporting peptide fragment couplings with low to null epimerization levels.<sup>15-20</sup> However, these examples remain relatively scarce. In this context, and based on our knowledge of peptide synthesis under solvent-less/solvent-free conditions by mechanochemical means,<sup>21-26</sup> we considered evaluating the capacity of ball-mills to reduce or eliminate

epimerization during C-term activation of peptide fragments, and to compare these results with the classical synthesis in solution.

The [2+1] coupling between Z-Ala-Phg-OH and HCl·H-Ile-OMe was considered as a probe of choice.<sup>27</sup> Indeed, phenylglycine (Phg) is known as being one of the most epimerization-prone amino acid,<sup>14, 17, 28-31</sup> while isoleucine is a hindered amino acid exhibiting low coupling speeds.<sup>32</sup> For this study, the target dipeptide Z-Ala-Phg-OH was prepared using Z/*t*Bu orthogonal strategy (see ESI for details). Then, Z-Ala-Phg-OH was reacted with HCl·H-Ile-OMe (1.5 equiv.), in the presence of EDC·HCl (1.2 equiv.), Oxyma (1.2 equiv.) and a small amount of DMF ( $\eta = 0.45 \mu\text{L}/\text{mg}$ ) in a 5 mL PTFE ball-mill reactor containing three stainless steel balls (5 mm diameter), for 10 min at 25 Hz (see ESI for details). Here, the amount of DMF was not sufficient to solubilize the reaction mixture but improved the homogeneity of the reaction mixture.<sup>33</sup> Although being a highly problematic solvent,<sup>34</sup> DMF is considered as the most widely used solvent for peptide synthesis.<sup>35-37</sup> It appeared as the liquid additive of choice to ensure a rigorous comparison between the ball-milling and the classical solution-phase approach. Following the same idea, the  $\eta$  parameter was calculated to facilitate the comparison of the solvent quantities that were used.<sup>38</sup> After reaction mixture recovery from the milling reactor and work-up, the corresponding tripeptide Z-Ala-Phg-Ile-OMe was isolated in 93% yield, with excellent purity and no detectable traces of the Z-Ala-D-Phg-Ile-OMe epimer as indicated by HPLC (Entry 1, Table 1;  $t_{\text{R}}(\text{LLL}) = 5.08 \text{ min}$  and  $t_{\text{R}}(\text{LDL}) = 5.20 \text{ min}$ , see ESI for details).



Entry	Reagents	Temp (°C)	t (min)	Yield (%)	Purity (%) <sup>a,b</sup>	LDL (%) <sup>b</sup>
1	EDC·HCl/Oxyma	33 (33)	10 (30)	93 (88)	> 99 (32)	< 1 (9)
2	EDC·HCl/HOBt·H <sub>2</sub> O	34 (34)	10 (30)	90 (90)	70 (48)	25 (35)
3	EDC·HCl/HOAt	34 (34)	10 (30)	88 (90)	95 (59)	< 1 (26)
4	DIC/HOAt	30 (31)	10 (40)	n.d. (n.d.) <sup>c</sup>	67 (39)	17 (33)
5	DIC/Oxyma	31 (31)	10 (40)	n.d. (n.d.) <sup>c</sup>	46 (< 10)	< 1 (n.d.)
6	HATU/Et <sub>3</sub> N	34 (34)	10 (60)	85 (88)	88 (58)	1 (< 1)
7	HBTU/Et <sub>3</sub> N	33 (33)	10 (20)	86 (82)	71 (55)	2 (9)

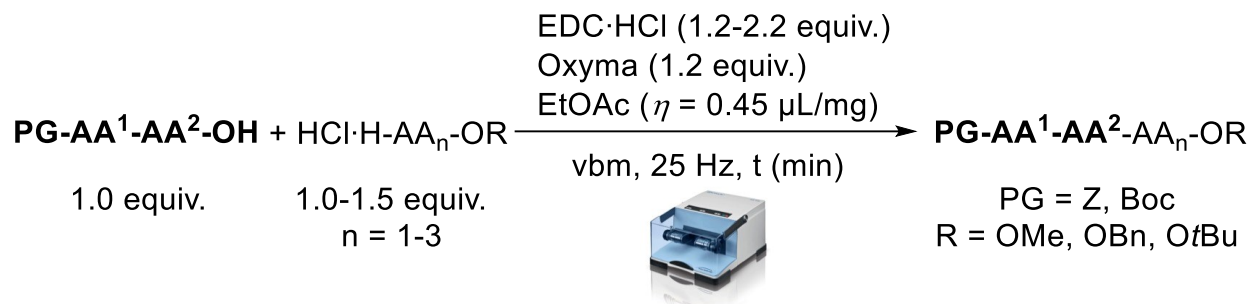
<sup>a</sup> Purity determined by HPLC and calculated as the surface of the LLL HPLC peak over all the other peaks, including the LDL peak. <sup>b</sup> Determined by HPLC. <sup>c</sup> n.d.: not determined.

Table 1: Comparative study between vibrating ball-milling (main figures) and solution synthesis (in parentheses) of Z-Ala-Phg-Ile-OMe

Ball-milling is known to induce a slight temperature raise of the milled materials.<sup>39-41</sup> As the temperature inside the reactor could have an influence on the reaction course, the temperature of the milled material was measured directly after milling. During the coupling under ball-milling conditions, the temperature reached 33 °C. The same temperature was applied to the synthesis of Z-Ala-Phg-Ile-OMe under classical magnetic stirring conditions, while using the minimal amount of DMF enabling proper mixing ( $\eta = 20 \text{ } \mu\text{L/mg}$ ). In these experimental conditions, Z-Ala-Phg-Ile-OMe was produced in 88% yield, 32% purity and 9% of the LDL epimer (Entry 1, figures in parentheses, Table 1). These results clearly showed that solvent-less ball-milling could minimize epimerization of a highly epimerization-prone C-term activated peptide fragment. Additionally, the coupling was complete after 10 min of ball-milling, while 30 min were required under classical solution conditions. This latter observation could be explained by the higher concentration

of the reaction mixture in the ball-mill compared to that in solution. Other coupling reagents and additives were screened to establish the generality of our observations. When couplings were performed in solution, the quantity of DMF was kept as low as possible to ensure maximal concentration of the reactants while enabling proper mixing under classical magnetic stirring ( $11 < \eta < 26 \mu\text{L}/\text{mg}$ ). When Oxyma was replaced with HOBt·H<sub>2</sub>O, ball-milling conditions led to 25% of the LDL epimer and 70% purity, while 35% of the same epimer and 48% purity were obtained for the synthesis in solution (Entry 2, Table 1). The use of HOAt gave better results than HOBt·H<sub>2</sub>O; only 1% of the LDL epimer was produced in the ball-milling conditions while 26% LDL epimer were obtained after synthesis in solution (Entry 3, Table 1). These results were in agreement with previous publications comparing HOBt·H<sub>2</sub>O and HOAt.<sup>42-43</sup> Using DIC with HOAt under ball-milling conditions furnished the tripeptide with 67% purity along with 17% of the LDL epimer, while synthesis in solution led to lower purity (39%) and higher epimerization (33%) (Entry 4, Table 1). These results obtained with DIC were not improved when replacing HOAt with Oxyma (Entry 5, Table 1). Concerning the stand-alone coupling reagents HBTU and HATU, their use induced low epimerization levels both by ball-milling and in solution (Entries 6 & 7, Table 1). Yet, the HPLC purity profiles of the products obtained by ball-milling were much better than when synthesized in solution. The comparison between ball-milling and solution-phase clearly showed the superiority of the mechanochemical approach over the classical synthesis in solution, since lower epimerization rates and better purity profiles were obtained with almost all tested coupling agents and additives, while yields remained comparable.

We next applied the best ball-milling experimental conditions (EDC·HCl/Oxyma) to the synthesis of other challenging peptides. Here, EtOAc was used instead of DMF to improve the overall environmental impact of our ball-milling conditions. Thus, ball-milling Z-Ala-Phg-OH with HCl·H-Phe-OMe produced the tripeptide Z-Ala-Phg-Phe-OMe in 89% yield with full conservation of the stereogenic centers (Entry 1, Table 2).



Entry	Peptides	Time (min)	Isolated yield (%)	de (%)
1	<b>Z-Ala-Phg-Phe-OMe</b>	20	89 <sup>a</sup>	> 99
2	<b>Z-Ala-D-Phg-Phe-OMe</b>	10	92 <sup>a</sup>	> 99
3	<b>Z-Ala-Cys(Bn)-Ala-OMe</b>	30	98 <sup>a</sup>	> 99
4	<b>Z-Ala-Cys(Bn)-Phe-OMe</b>	20	94 <sup>a</sup>	> 99
5	<b>Z-Phe-Val-Cys(Bn)-OMe</b>	30	98 <sup>a,b</sup>	> 99
6	<b>Z-Phe-D-Val-Cys(Bn)-OMe</b>	30	97 <sup>a</sup>	> 99
7	<b>Z-Phe-Val-Ser(<i>t</i>Bu)-OtBu</b>	30	98 <sup>a,b</sup>	> 99
8	<b>Z-Phe-D-Val-Ser(<i>t</i>Bu)-OtBu</b>	20	97 <sup>a</sup>	> 99
9	<b>Boc-Trp-Phe-Glu(Bn)-OBn</b>	20	92 <sup>a</sup>	> 99
10	<b>Boc-Trp-Phe-Gly-OBn</b>	10	84 <sup>a</sup>	> 99
11	<b>Boc-Trp-D-Phe-Gly-OBn</b>	10	95 <sup>a</sup>	> 99
12	<b>Z-Phe-Val-Leu<sub>2</sub>-OBn</b>	25	95 <sup>c</sup>	> 99
13	<b>Z-Phe-D-Val-Leu<sub>2</sub>-OBn</b>	25	91 <sup>c</sup>	98
14	<b>Z-Phe-Val-Leu<sub>3</sub>-OBn</b>	30	93 <sup>c,d</sup>	> 99
15	<b>Z-Phe-D-Val-Leu<sub>3</sub>-OBn</b>	30	91 <sup>c,d</sup>	99

<sup>a</sup> 1.5 equiv. of HCl·H-AA<sub>n</sub>-OR were used. <sup>b</sup> 1.2 equiv. of EDC·HCl were used. <sup>c</sup> 1.0 equiv. of HCl·H-AA<sub>n</sub>-OR were used. <sup>d</sup> 2.2 equiv. of EDC·HCl and  $\eta$  (EtOAc) = 0.9  $\mu\text{L}/\text{mg}$  were used.

Table 2: Scope of the peptide couplings by ball-milling

When Z-Ala-D-Phg-OH was used instead of Z-Ala-L-Phg-OH, the tripeptide Z-Ala-D-Phg-Phe-OMe was isolated with a similar yield of 92% and excellent purity (Entry 2, Table 2). Cysteine, which is another epimerization-prone amino acid,<sup>14, 44</sup> was next tested under

the same experimental conditions. Thus, Z-Ala-Cys(Bn)-Ala-OMe and Z-Ala-Cys(Bn)-Phe-OMe were synthesized from Z-Ala-Cys(Bn)-OH. In both cases, tripeptides were obtained in excellent yields (> 94%) without any detectable epimerization (Entries 3 & 4, Table 2). Like isoleucine, valine is a  $\beta$ -branched amino acid known to be more difficult for coupling than most of other amino acids.<sup>32</sup> Yet, the reaction between Z-Phe-Val-OH and HCl·H-Cys(Bn)-OMe led to the desired tripeptide, Z-Phe-Val-Cys(Bn)-OMe, in almost quantitative yield after 30 minutes ball-milling (Entry 5, Table 2). Importantly, no trace of the LDL epimer could be detected by HPLC analysis. Similar results were obtained starting with the Z-Phe-D-Val-OH (Entry 6, Table 2). When HCl·H-Cys(Bn)-OMe was replaced with HCl·H-Ser(*t*Bu)-O*t*Bu, excellent yields were obtained, again with no epimerization (Entries 7 & 8, Table 2). Of note, the *t*-butyl ester was stable in these experimental conditions. In classical peptide synthesis in solution, Z and Boc *N*-protecting groups are commonly used. In order to evaluate the coupling of Boc-protected fragments, Boc-Trp-Phe-Glu(OBn)-OBn, Boc-Trp-Phe-Gly-OBn, and Boc-Trp-D-Phe-Gly-OBn were synthesized and successfully isolated in good to excellent yields (84-95%), without any detectable epimerization (Entries 9-11, Table 2). These results supported that ball-milling conditions were compatible with Boc *N*-protecting groups, without affecting the isolated yields and diastereomeric excesses of the expected products. The presence of benzyl groups was not an obstacle to the success of these syntheses.

After clearly demonstrating the efficiency of our method in epimerization-free [2+1] peptide couplings, we aimed at demonstrating that peptide fragment couplings mediated by ball-milling could be applied to longer peptide fragments. For this, [2+2] and [2+3] couplings involving peptide fragments bearing the hindered valine at C-term were performed. When the optimal experimental reaction conditions were applied to the coupling of Z-Phe-Val-OH with HCl·H-Leu<sub>2</sub>-OBn, the expected tetrapeptide was obtained in 95% yield without epimerization (Entry 12, Table 2). The same conditions applied to the synthesis of the diastereomer Z-Phe-D-Val-Leu<sub>2</sub>-OBn yielded the desired tetrapeptide in 91%, yet containing 2% of the epimer (Entry 13, Table 2). To our delight, when the tripeptide HCl·H-Leu<sub>3</sub>-OBn was reacted with Z-Phe-Val-OH and Z-Phe-D-Val-OH, the corresponding pentapeptides Z-Phe-Val-Leu<sub>3</sub>-OBn and Z-Phe-D-Val-Leu<sub>3</sub>-OBn were successfully isolated with high yields (> 91%) and no to negligible level of epimerization



(Entries 14 & 15, Table 2). So far, the longest peptide synthesized by ball-milling was the pentapeptide Leu-enkephalin.<sup>22</sup> The successful synthesis of these two pentapeptides by a fragment coupling approach consolidates the potential of mechanochemistry to produce oligopeptides with high efficiency. Overall, the lower levels of epimerization observed under ball-milling conditions could find explanations in the concentrations of the reaction mixtures. Indeed, epimerization during peptide couplings occurs either through direct  $\alpha$ H-abstraction in basic medium and/or through the formation of an oxazolone ring during activation.<sup>14</sup> Of note, the latter mechanism is an intramolecular process favored in diluted conditions, whereas “direct” peptide coupling (in other words, acylation of the free amine) is an intermolecular process, comparatively favored in concentrated conditions such as those enabled by ball-milling. Consequently, our results suggest that the absence of epimerization could be explained by the highly concentrated reaction conditions enabled by ball-milling, that would prevent the transient formation of an oxazolone ring.

To conclude, using the combination of EDC·HCl, Oxyma and small amounts of a liquid additive (DMF or EtOAc) under ball-milling conditions allowed to perform [2+1], [2+2], and [2+3] peptide couplings with high yields and, if any, very low epimerization. Noteworthy, very good results were obtained with peptide fragments containing highly epimerization-prone and/or highly hindered amino acids at C-term such as phenylglycine, cysteine and valine. Ball-milling was clearly identified as the key element to obtain both high yield and purity along with low epimerization. Indeed, the ball-milling conditions proved to be superior to the classical solution synthesis approach on a various array of widely used coupling agents. These results open avenues for the development of highly efficient, convergent and flexible peptide synthesis strategies based on peptide fragment couplings. Further investigations aiming at *i*) deciphering the mechanism enabling epimerization suppression during ball-milling when compared to synthesis in solution *ii*) applying this strategy to longer peptide fragments are currently being explored in our laboratory and will be reported in due course.

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Université de Montpellier, Centre National de la Recherche Scientifique (CNRS), and LabEx CheMISyst (through ANR programme ANR-10-LABX-05-01) are acknowledged for financial support. Monique Calmès is acknowledged for assistance in chiral HPLC analysis.

## Abbreviations

DIC	<i>N,N'</i> -diisopropylcarbodiimide
EDC·HCl	<i>N</i> -(3-Dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide hydrochloride
EMA	European Medicines Agency
FDA	Food and Drug Administration
HATU	<i>N</i> -[(Dimethylamino)-1 <i>H</i> -1,2,3-triazolo-[4,5- <i>b</i> ]pyridin-1-ylmethylene]- <i>N</i> -methylmethanaminium hexafluorophosphate <i>N</i> -oxide
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
HOBT·H <sub>2</sub> O	1-Hydroxybenzotriazole hydrate
Oxyma	Ethyl hydroxyiminocyanoacetate
vbm	Vibrating ball-milling

## References

- (1) Xia, S.; Liu, M.; Wang, C.; Xu, W.; Lan, Q.; Feng, S.; Qi, F.; Bao, L.; Du, L.; Liu, S.; Qin, C.; Sun, F.; Shi, Z.; Zhu, Y.; Jiang, S.; Lu, L. Inhibition of SARS-CoV-2 (previously 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting its spike protein that harbors a high capacity to mediate membrane fusion. *Cell Res.* **2020**, *30*, 343-355, DOI: 10.1038/s41422-020-0305-x.
- (2) Liu, C.; Zhou, Q.; Li, Y.; Garner, L. V.; Watkins, S. P.; Carter, L. J.; Smoot, J.; Gregg, A. C.; Daniels, A. D.; Jervy, S.; Albaiu, D. Research and Development on Therapeutic Agents and Vaccines for COVID-19 and Related Human Coronavirus Diseases. *ACS Cent. Sci.* **2020**, *6*, 315-331, DOI: 10.1021/acscentsci.0c00272.
- (3) Raines, R. T.; Wennemers, H. Peptides on the Rise. *Acc. Chem. Res.* **2017**, *50*, 2419, DOI: 10.1021/acs.accounts.7b00471.
- (4) Lau, J. L.; Dunn, M. K. Therapeutic peptides: Historical perspectives, current development trends, and future directions. *Biorg. Med. Chem.* **2018**, *26*, 2700-2707, DOI: 10.1016/j.bmc.2017.06.052.
- (5) Henninot, A.; Collins, J. C.; Nuss, J. M. The Current State of Peptide Drug Discovery: Back to the Future? *J. Med. Chem.* **2018**, *61*, 1382-1414, DOI: 10.1021/acs.jmedchem.7b00318.
- (6) Impact Story: Developing the Tools to Evaluate Complex Drug Products: Peptides. <https://www.fda.gov/drugs/regulatory-science-action/impact-story-developing-tools-evaluate-complex-drug-products-peptides> (accessed 11/05).

- (7) Al Shaer, D.; Al Musaimi, O.; Albericio, F.; de la Torre, B. G. 2019 FDA TIDES (Peptides and Oligonucleotides) Harvest. *Pharmaceuticals* **2020**, *13*, 40, DOI: 10.3390/ph13030040.
- (8) Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisky, M. Synthetic therapeutic peptides: science and market. *Drug Discov. Today* **2010**, *15*, 40-56, DOI: 10.1016/j.drudis.2009.10.009.
- (9) Martinez, J.; Fehrentz, J.-A. From JMV-1843 to Macrilen. *Aust. J. Chem.* **2020**, *73*, 334-337, DOI: 10.1071/ch19530.
- (10) Lax, E. R.; Shah, T. In *Peptide Therapeutics: Strategy and Tactics for Chemistry, Manufacturing, and Controls*, The Royal Society of Chemistry: **2019**, 151-193, DOI: 10.1039/9781788016445-00151.
- (11) Raibaut, L.; Ollivier, N.; Melnyk, O. Sequential native peptide ligation strategies for total chemical protein synthesis. *Chem. Soc. Rev.* **2012**, *41*, 7001-7015, DOI: 10.1039/c2cs35147a.
- (12) Conibear, A. C.; Watson, E. E.; Payne, R. J.; Becker, C. F. W. Native chemical ligation in protein synthesis and semi-synthesis. *Chem. Soc. Rev.* **2018**, *47*, 9046-9068, DOI: 10.1039/C8CS00573G.
- (13) Agouridas, V.; El Mahdi, O.; Diemer, V.; Cargoët, M.; Monbaliu, J.-C. M.; Melnyk, O. Native Chemical Ligation and Extended Methods: Mechanisms, Catalysis, Scope, and Limitations. *Chem. Rev.* **2019**, *119*, 7328-7443, DOI: 10.1021/acs.chemrev.8b00712.
- (14) Yang, Y. In *Side Reactions in Peptide Synthesis*, Academic Press: **2016**, 257-292, DOI: 10.1016/B978-0-12-801009-9.00011-2.
- (15) Hiebl, J.; Baumgartner, H.; Bernwieser, I.; Blanka, M.; Bodenteich, M.; Leitner, K.; Rio, A.; Rovenszky, F.; Alberts, D. P.; Bhatnagar, P. K.; Banyard, A. F.; Baresch, K.; Esch, P. M.; Kollmann, H.; Mayrhofer, G.; Weihtrager, H.; Welz, W.; Winkler, K.; Chen, T.; Patel, R.; Lantos, I.; Stevenson, D.; Tubman, K. D.; Undheim, K. Large-scale synthesis of hematoregulatory nonapeptide SK&F 107647 by fragment coupling. *J. Peptide Res.* **1999**, *54*, 54-65, DOI: 10.1034/j.1399-3011.1999.00089.x.
- (16) Spengler, J.; Ruíz-Rodríguez, J.; Yraola, F.; Royo, M.; Winter, M.; Burger, K.; Albericio, F. A Novel Protecting/Activating Strategy for  $\beta$ -Hydroxy Acids and Its Use in Convergent Peptide Synthesis. *J. Org. Chem.* **2008**, *73*, 2311-2314, DOI: 10.1021/jo7025788.
- (17) Popovic, S.; Bieräugel, H.; Detz, R. J.; Kluwer, A. M.; Koole, J. A. A.; Streefkerk, D. E.; Hiemstra, H.; van Maarseveen, J. H. Epimerization-Free C-Terminal Peptide Activation. *Chem. Eur. J.* **2013**, *19*, 16934-16937, DOI: 10.1002/chem.201303347.
- (18) Zhang, T.; Chen, Z.; Tian, Y.; Han, B.; Zhang, N.; Song, W.; Liu, Z.; Zhao, J.; Liu, J. Kilogram-Scale Synthesis of Osteogenic Growth Peptide (10–14) Using a Fragment Coupling Approach. *Org. Process Res. Dev.* **2015**, *19*, 1257-1262, DOI: 10.1021/acs.oprd.5b00004.

- (19) Zhang, T.; Song, W.; Zhao, J.; Liu, J. Full Solution-Phase Synthesis of Acetyl Hexapeptide-3 by Fragments Coupling Strategy. *Ind. Eng. Chem. Res.* **2017**, *56*, 11697-11704, DOI: 10.1021/acs.iecr.7b03299.
- (20) Gurjar, M. K.; Tripathy, N. K.; Pramanik, C. M.; Deshmukh, S. S.; Mahajan, U. S.; Deshpande, A. P. Process for preparation of octreotide acetate via an improved 4+4 soln. phase synthesis of octreotide. WO2017175107A1, 2017.
- (21) Declerck, V.; Nun, P.; Martinez, J.; Lamaty, F. Solvent-Free Synthesis of Peptides. *Angew. Chem. Int. Ed.* **2009**, *48*, 9318-9321, DOI: 10.1002/anie.200903510.
- (22) Bonnamour, J.; Métro, T.-X.; Martinez, J.; Lamaty, F. Environmentally benign peptide synthesis using liquid-assisted ball-milling: application to the synthesis of Leu-enkephalin. *Green Chem.* **2013**, *15*, 1116-1120, DOI: 10.1039/c3gc40302e.
- (23) Porte, V.; Thiolo, M.; Pigoux, T.; Métro, T.-X.; Martinez, J.; Lamaty, F. Peptide Mechanosynthesis by Direct Coupling of N-Protected  $\alpha$ -Amino Acids with Amino Esters. *Eur. J. Org. Chem.* **2016**, 3505-3508, DOI: 10.1002/ejoc.201600617.
- (24) Maurin, O.; Verdié, P.; Subra, G.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide synthesis: ball-milling, in solution, or on solid support, what is the best strategy? *Beilstein J. Org. Chem.* **2017**, *13*, 2087-2093, DOI: 10.3762/bjoc.13.206.
- (25) Pétry, N.; Benakki, H.; Clot, E.; Retailleau, P.; Guenoun, F.; Asserar, F.; Sekkat, C.; Métro, T.-X.; Martinez, J.; Lamaty, F. A mechanochemical approach to access the proline–proline diketopiperazine framework. *Beilstein J. Org. Chem.* **2017**, *13*, 2169-2178, DOI: 10.3762/bjoc.13.217.
- (26) Yeboue, Y.; Gallard, B.; Le Moigne, N.; Jean, M.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide couplings by reactive extrusion: solid-tolerant and free from carcinogenic, mutagenic and reprotoxic chemicals. *ACS Sustainable Chem. Eng.* **2018**, *6*, 16001-16004, DOI: 10.1021/acssuschemeng.8b04509.
- (27) For sake of clarity, absolute configuration indications of L amino acids have been omitted throughout the manuscript. Only D configurations have been indicated. Thus, Z-Ala-Phg-OH corresponds to Z-L-Ala-L-Phg-OH.
- (28) Smith, G. G.; Sivakua, T. Mechanism of the racemization of amino acids. Kinetics of racemization of arylglycines. *J. Org. Chem.* **1983**, *48*, 627-634, DOI: 10.1021/jo00153a001.
- (29) Elsayy, M. A.; Hewage, C.; Walker, B. Racemisation of N-Fmoc phenylglycine under mild microwave-SPPS and conventional stepwise SPPS conditions: attempts to develop strategies for overcoming this. *J. Pept. Sci.* **2012**, *18*, 302-311, DOI: 10.1002/psc.2398.
- (30) Fuse, S.; Mifune, Y.; Nakamura, H.; Tanaka, H. Total synthesis of feglymycin based on a linear/convergent hybrid approach using micro-flow amide bond formation. *Nat. Commun.* **2016**, *7*, 13491, DOI: 10.1038/ncomms13491.
- (31) Liang, C.; Behnam, M. A. M.; Sundermann, T. R.; Klein, C. D. Phenylglycine racemization in Fmoc-based solid-phase peptide synthesis: Stereochemical stability is achieved by choice of reaction conditions. *Tetrahedron Lett.* **2017**, *58*, 2325-2329, DOI: 10.1016/j.tetlet.2017.04.047.

- (32) Ostresh, J. M.; Winkle, J. H.; Hamashin, V. T.; Houghten, R. A. Peptide libraries: Determination of relative reaction rates of protected amino acids in competitive couplings. *Biopolymers* **1994**, *34*, 1681-1689, DOI: 10.1002/bip.360341212.
- (33) Adding a liquid additive to a mechanochemical reaction, known as Liquid Assisted Grinding (LAG), is known to have beneficial effects during peptide couplings under mechanochemical conditions. See references: a) Bowmaker, G. A., *Chem. Commun.* **2013**, *49*, 334-348; b) Friščić, T.; Reid, D. G.; Halasz, I.; Stein, R. S.; Dinnebier, R. E.; Duer, M. J., *Angew. Chem. Int. Ed.* **2010**, *49*, 712-715 and references 22) and 23).
- (34) *N,N*-dimethylformamide (DMF) is classified as presenting reproductive toxicity according to Regulation (EC) No 1272/2008.
- (35) MacMillan, D. S.; Murray, J.; Sneddon, H. F.; Jamieson, C.; Watson, A. J. B. Evaluation of alternative solvents in common amide coupling reactions: replacement of dichloromethane and *N,N*-dimethylformamide. *Green Chem.* **2013**, *15*, 596-600, DOI: 10.1039/c2gc36900a.
- (36) Jad, Y. E.; Acosta, G. A.; Khattab, S. N.; de la Torre, B. G.; Govender, T.; Kruger, H. G.; El-Faham, A.; Albericio, F. Peptide synthesis beyond DMF: THF and ACN as excellent and friendlier alternatives. *Org. Biomol. Chem.* **2015**, *13*, 2393-2398, DOI: 10.1039/c4ob02046d.
- (37) Pawlas, J.; Rasmussen, J. H. ReGreen SPPS: enabling circular chemistry in environmentally sensible solid-phase peptide synthesis. *Green Chem.* **2019**, *21*, 5990-5998, DOI: 10.1039/c9gc02775k.
- (38) The  $\eta$  parameter is defined by the ratio of the volume of liquid (in  $\mu\text{l}$ ) to the total mass of solids (in mg). See reference: Friščić, T.; Childs, S. L.; Rizvi, S. A. A.; Jones, W., *CrystEngComm* **2009**, *11*, 418-426.
- (39) Kulla, H.; Wilke, M.; Fischer, F.; Rollig, M.; Maierhofer, C.; Emmerling, F. Warming up for mechanosynthesis - Temperature development in ball mills during synthesis. *Chem. Commun.* **2017**, *53*, 1664-1667, DOI: 10.1039/C6CC08950J.
- (40) Užarević, K.; Ferdelji, N.; Mrla, T.; Julien, P.; Halasz, B.; Friščić, T.; Halasz, I. Enthalpy vs. friction: Heat flow modelling of unexpected temperature profiles in mechanochemistry of metal-organic frameworks. *Chem. Sci.* **2018**, *9*, 2525-2532, DOI: 10.1039/C7SC05312F.
- (41) Ardila-Fierro, K. J.; Lukin, S.; Etter, M.; Užarević, K.; Halasz, I.; Bolm, C.; Hernández, J. G. Direct Visualization of a Mechanochemically Induced Molecular Rearrangement. *Angew. Chem. Int. Ed.* **2020**, DOI: 10.1002/anie.201914921.
- (42) El-Faham, A.; Albericio, F. Peptide Coupling Reagents, More than a Letter Soup. *Chem. Rev.* **2011**, *111*, 6557-6602, DOI: 10.1021/cr100048w.
- (43) Subirós-Funosas, R.; Prohens, R.; Barbas, R.; El-Faham, A.; Albericio, F. Oxyma: An Efficient Additive for Peptide Synthesis to Replace the Benzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion. *Chem. Eur. J.* **2009**, *15*, 9394-9403, DOI: 10.1002/chem.200900614.

(44) Han, Y.; Albericio, F.; Barany, G. Occurrence and Minimization of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis. *J. Org. Chem.* **1997**, 62, 4307-4312, DOI: 10.1021/jo9622744.

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# **Epimerization-free C-term Activation of Peptide Fragments by Ball-Milling**

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## General information

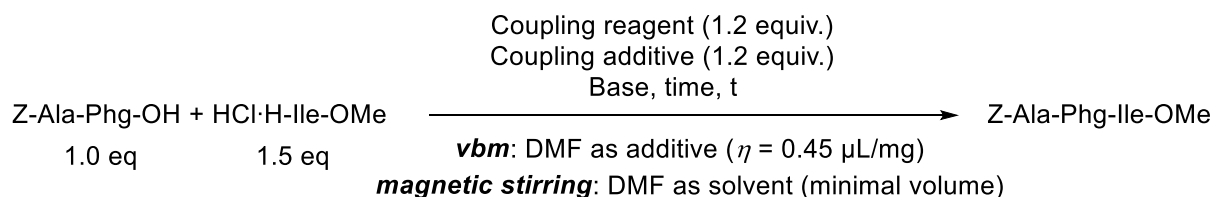
All reagents were purchased from Sigma Aldrich, TCI chemicals, Iris Biotech GmbH and used without further purification. The milling treatments were carried out in a vibrating ball-mill Retsch Mixer Mill 200 or Mixer Mill 400.  $\eta$  is defined as the ratio of added liquid volume to the total mass of reagents.<sup>1</sup> It is expressed in  $\mu\text{L}/\text{mg}$ . NMR analyses were performed at the "Plateforme Technologique Laboratoire de Mesures Physiques" (IBMM, Université de Montpellier).  $^1\text{H}$  NMR spectra were recorded on a Bruker Avance III HD 400 MHz spectrometer and are reported in ppm using solvent as an internal standard ( $\text{CDCl}_3$  at 7.26 ppm,  $\text{CD}_3\text{OD}$  at 3.31 ppm and  $\text{DMSO}-d_6$  at 2.50 ppm). Data are reported as s = singlet, d = doublet, t = triplet, qd = quadruplet, qt = quintuplet, m = multiplet; coupling constant in Hz; integration.  $^{13}\text{C}$  NMR spectra were recorded on a Bruker Avance III HD 101 MHz spectrometer and are reported in ppm using solvent as an internal standard ( $\text{CDCl}_3$  at 77.16 ppm,  $\text{CD}_3\text{OD}$  at 49.00 ppm and  $\text{DMSO}-d_6$  at 39.52 ppm). Mass spectra were obtained by LC-MS with ESI using a Water Alliance 2695 as LC, coupled to a Waters ZQ spectrometer with electrospray source, a simple quadrupole analyzer and a UV Waters 2489 detector. HRMS analyses were performed on UPLC Acquity H-Class from Waters hyphenated to a Synapt G2-S mass spectrometer with a dual ESI source from Waters. Diastereomeric excesses of Z-Ala-Phg-Ile-OMe were measured using an Agilent technologies 1220 Infinity LC using a Chromolith® high resolution RP-18e 50-4.6 mm column. Diastereomeric excesses of Z-Ala-Cys(Bn)-Ala-OMe and Z-Ala-Cys(Bn)-Phe-OMe were measured using a Beckman Coulter System Gold 126 Module HPLC machine and System Gold 168 Detector with 4.6 mm Diacel Chiralpak AS-H columns using *n*-hexane and isopropanol as solvents. Diastereomeric excesses of all other compounds were measured at the "Plateforme de chromatographie chirale" of the Institut des Sciences Moléculaires de Marseille (iSm2, Marseille, France), on an Agilent 1260 Infinity unit (pump G1311B, autosampler G1329B, DAD G1315D). These analyses were performed by using either Chiralpak IA, ID, IE or IF analytical columns (250x4.6 mm, 5  $\mu\text{m}$ ) from Chiral Technologies Europe (Illkirch, France). HPLC grade heptane and ethanol were used as solvents, detection at 205 nm, 220 nm and 230 nm. Diastereomeric ratios were calculated from the area of the peaks on the UV chromatogram, assuming that the molar absorption coefficient were the same for both diastereomers.

Oxyrna stands for ethyl (hydroxyimino)cyanoacetate, EDC·HCl for *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride.

For sake of clarity, absolute configuration indications of L amino acids have been omitted throughout the manuscript. Only D configurations have been indicated. Thus, Z-Ala-Phg-OH corresponds to Z-L-Ala-L-Phg-OH.

## Operating procedures and analytical data

- ❖ Comparative study between ball-milling and solution processes for the synthesis of Z-Ala-Phg-Ile-OMe



Reactions were performed on a total mass of reagents of 50 mg.

- **Procedure 1:** reactions performed in a vibrating ball-mill (vbm)

Z-Ala-Phg-OH (1.0 equiv.), HCl·H-Ile-OMe (1.5 equiv.), coupling additive (if used, cf Table 1 of the main text, 1.2 equiv.), Et<sub>3</sub>N (if used, cf Table 1 of the main text, 1.2 equiv.) and DMF (23  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 5 mL PTFE grinding jar with three stainless steel balls (5 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, the coupling reagent (1.2 equiv.) was introduced and the jar was closed and subjected to grinding in the mixer mill operated at 25 Hz until completion of the reaction. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL), brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to give the desired product.

The diastereomeric excess of Z-Ala-Phg-Ile-OMe was measured, before and after the work-up procedure, on an Agilent technologies 1220 Infinity LC using a Chromolith® high resolution RP-18e 50-4.6 mm column and a linear gradient of 0 to 58% CH<sub>3</sub>CN/0.1% TFA in H<sub>2</sub>O/0.1%TFA over 6 min, detection at 214 nm. Flow rate: 3 mL/min.

- **Procedure 2:** reactions performed in solution under classical magnetic stirring

Z-Ala-Phg-OH (1.0 equiv.), HCl·H-Ile-OMe (1.5 equiv.), coupling additive (if used, cf Table 1 of the main text, 1.2 equiv.) and Et<sub>3</sub>N (if used, cf Table 1 of the main text, 1.2 equiv.) were introduced in a round-bottomed flask. The minimum volume of DMF enabling complete dissolution of the reagents ( $11 < \eta < 20$   $\mu$ L/mg) was added and the reaction mixture was stirred at room temperature for 1 min. Then, coupling reagent (1.2 equiv.) were added to the mixture. After completion of the reaction, EtOAc (10 mL) was added to the reaction mixture and the organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL), brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to give the desired product.

The diastereomeric excess of Z-Ala-Phg-Ile-OMe was measured, before and after the washing steps, on an Agilent technologies 1220 Infinity LC using a Chromolith® high resolution RP-18e 50-4.6 mm column and a linear gradient of 0 to 58% CH<sub>3</sub>CN/0.1% TFA in H<sub>2</sub>O/0.1%TFA over 6 min, detection at 214 nm. Flow rate: 3 mL/min.

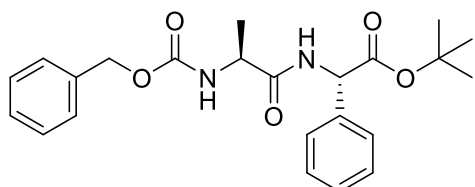
The results of these experiments are summarized in the following table. Main figures are corresponding to the experiments performed in the vibrating ball-mill, those in parenthesis are the results corresponding to the experiments realised in solution:

Entry	Reagents	Temp (°C)	t (min)	Yield (%)	Purity (%) <sup>a,b</sup>	LDL (%) <sup>b</sup>
1	EDC·HCl/Oxyma	33 (33)	10 (30)	93 (88)	> 99 (32)	< 1 (9)
2	EDC·HCl/HOBt·H <sub>2</sub> O	34 (34)	10 (30)	90 (90)	70 (48)	25 (35)
3	EDC·HCl/HOAt	34 (34)	10 (30)	88 (90)	95 (59)	< 1 (26)
4	DIC/HOAt	30 (31)	10 (40)	n.d. (n.d.)	67 (39)	17 (33)
5	DIC/Oxyma	31 (31)	10 (40)	n.d. (n.d.)	46 (< 10)	< 1 (n.d.)
6	HATU/Et <sub>3</sub> N	34 (34)	10 (60)	85 (88)	88 (58)	1 (< 1)
7	HBTU/Et <sub>3</sub> N	33 (33)	10 (20)	86 (82)	71 (55)	2 (9)

<sup>a</sup> Purity determined by HPLC and calculated as the surface of the LLL HPLC peak over all the other peaks, including the LDL peak. <sup>b</sup> Determined by HPLC. n.d.: not determined.

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.46–7.19 (11H), 6.39 (d, *J* = 7.7 Hz, 1H), 5.50 (d, *J* = 6.9 Hz, 1H), 5.42 (br d, *J* = 4.6 Hz, 1H), 5.08 (s, 2H), 4.53 (dd, *J* = 8.3, 4.8 Hz, 1H), 4.39–4.27 (m, 1H), 3.62 (s, 3H), 1.96–1.82 (1H), 1.45–1.30 (4H), 1.22–1.08 (m, 1H), 0.95–0.82 (6H); **<sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)** δ (ppm) 171.9, 171.8, 169.5, 156.0, 137.2, 136.3, 129.2, 128.72, 128.67, 128.3, 128.2, 127.3, 127.2, 67.1, 57.3, 57.0, 52.3, 50.6, 37.9, 25.3, 19.0, 15.5, 11.7; **HRMS (ESI)**: calcd for C<sub>26</sub>H<sub>34</sub>N<sub>3</sub>O<sub>6</sub> = 484.2448 [M+H]<sup>+</sup>; found = 484.2452.

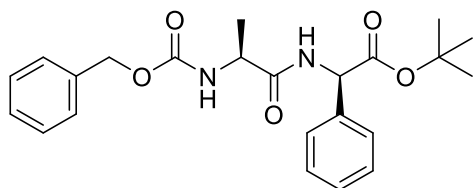
- Z-Ala-Phg-OfBu<sup>2</sup> [860013-16-7]



Z-Ala-OH (412.6 mg, 1.849 mmol), HCl·H-Phg-OfBu (540.6 mg, 2.218 mmol, 1.2 equiv.), Oxyma (315.2 mg, 2.218 mmol, 1.2 equiv.), NaH<sub>2</sub>PO<sub>4</sub> (887.1 mg, 7.394 mmol, 4.0 equiv.) and EtOAc (1.13 mL,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC (393  $\mu$ L, 2.218 mmol, 1.2 equiv.) was introduced and the jar was closed and subjected to grinding for 20 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionised water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 10 mL), 1N aqueous NaOH solution (2 x 10 mL) and brine (1 x 15 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 732 mg of the desired product as a yellow solid (96% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.39–7.25 (10H), 7.20 (br s, 1H), 5.47 (br d, *J* = 7.1 Hz, 1H), 5.41 (d, *J* = 7.3 Hz, 1H), 5.07 (s, 2H), 4.35 (br s, 1H), 1.46–1.34 (12H), **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 171.5, 169.9, 155.9, 136.9, 136.3, 128.8, 128.5, 128.2, 128.1, 128.0, 127.0, 82.7, 66.9, 57.0, 50.4, 27.8, 18.8; **MS (ESI)**: *m/z* = 435.0 [M+Na]<sup>+</sup>

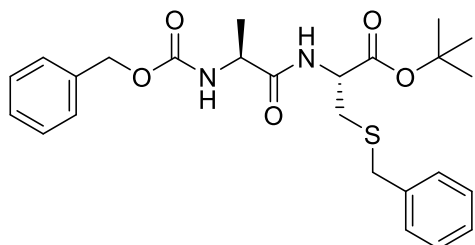
- Z-Ala-D-Phg-OfBu



Z-Ala-OH (682.3 mg, 3.056 mmol), H-D-Phg-OfBu (760.2 mg, 3.668 mmol, 1.2 equiv.), Oxyma (512.3 mg, 3.668 mmol, 1.2 equiv.), NaH<sub>2</sub>PO<sub>4</sub> (1.467 mg, 12.23 mmol, 4.0 equiv.) and EtOAc (1.8 mL,  $\eta$  = 0.45 mL/g) were introduced in a 25 mL PTFE grinding jar with one PTFE ball (20 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC (649.2  $\mu$ L, 3.668 mmol, 1.2 equiv.) was introduced and the jar was closed and subjected to grinding for 15 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 15 mL), 1N aqueous NaOH solution (2 x 15 mL) and brine (1 x 20 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 1.06 g of the desired product as an orange solid (84% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.37–7.26 (10H), 7.12 (br s, 1H), 5.44–5.37 (2H), 5.12 (s, 2H), 4.34 (br s, 1H), 1.42–1.33 (12H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 171.5, 169.7, 156.0, 137.0, 128.9, 128.7, 128.33, 128.28, 128.2, 127.1, 82.9, 67.2, 57.1, 50.5, 27.9, 18.7; **HRMS (ESI)**: calcd for C<sub>23</sub>H<sub>28</sub>N<sub>2</sub>O<sub>5</sub>Na = 435.1896 [M+Na]<sup>+</sup>; found = 435.1912.

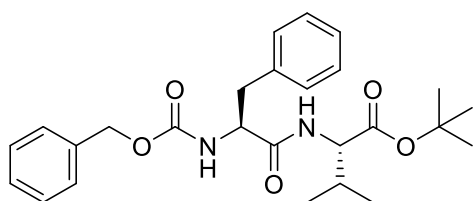
- Z-Ala-Cys(Bn)-OtBu



Z-Ala-OH (611.2 mg, 2.738 mmol), H-Cys(Bn)-OtBu (1098.1 mg, 4.107 mmol, 1.5 equiv.), Oxyma (466.9 mg, 3.285 mmol, 1.2 equiv.), NaH<sub>2</sub>PO<sub>4</sub> (1313.9 mg, 10.951 mmol, 4.0 equiv.) and EtOAc (1.8 mL,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 25 mL PTFE grinding jar with one PTFE ball (20 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC (581.5  $\mu$ L, 3.285 mmol, 1.2 equiv.) was introduced and the jar was closed and subjected to grinding for 10 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionised water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 20 mL), 1N aqueous NaOH solution (2 x 20 mL) and brine (1 x 30 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 1223 mg of the desired product as a yellow solid (95% yield).

**<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.37–7.20 (m, 10H), 6.62 (d,  $J$  = 5.4 Hz, 1H), 5.34 (d,  $J$  = 6.0 Hz, 1H), 5.08 (s, 2H), 4.62 (dd,  $J$  = 12.7, 5.2 Hz, 1H), 4.23 (m, 1H), 3.67 (s, 2H), 2.86 (dd,  $J$  = 13.8, 4.7 Hz, 1H), 2.80 (dd,  $J$  = 13.8, 5.7 Hz, 1H), 1.42 (s, 9H), 1.36 (d,  $J$  = 7.0 Hz, 3H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 172.1, 169.4, 155.9, 138.0, 136.3, 129.1, 128.7, 128.6, 128.3, 128.2, 127.3, 82.9, 67.1, 52.4, 50.5, 36.9, 33.7, 28.1, 18.9; **HRMS (ESI)**: calcd for C<sub>25</sub>H<sub>33</sub>N<sub>2</sub>O<sub>5</sub>S = 473.2110 [M+H]<sup>+</sup>; found = 473.2107.

- Z-Phe-Val-OtBu [14470-31-6]

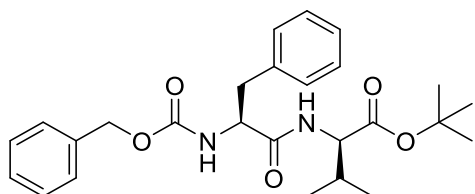


Z-Phe-OH (825.4 mg, 2.757 mmol), HCl·H-Val-OtBu (867.4 mg, 4.136 mmol, 1.5 equiv.), Oxyma (470.2 mg, 3.309 mmol, 1.2 equiv.), NaH<sub>2</sub>PO<sub>4</sub> (1.323 g, 11.030 mmol, 4 equiv.) and EtOAc (1.8 mL,  $\eta$  = 0.45 mL/g) were introduced in a 25 mL PTFE grinding jar with one PTFE ball (20 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC (585.7  $\mu$ L, 3.309 mmol, 1.2 equiv.) was added and the jar was closed and subjected to grinding for 10 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 15 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 15 mL), 1N aqueous NaOH solution (2 x 15 mL) and brine (1 x 15 mL) and

then dried over  $\text{MgSO}_4$ , filtered and concentrated under reduced pressure to afford 1.204 g of the desired product as a yellow solid (96% yield).

**$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )**  $\delta$  (ppm) 7.37–7.15 (10H), 6.35 (d,  $J$  = 8.0 Hz, 1H), 5.37 (d,  $J$  = 7.1 Hz, 1H), 5.11 (d,  $J$  = 12.4 Hz, 1H), 5.07 (d,  $J$  = 12.4 Hz, 1H), 4.47 (br d,  $J$  = 6.9 Hz, 1H), 4.35 (dd,  $J$  = 8.5, 4.6 Hz, 1H), 3.09 (d,  $J$  = 6.6 Hz, 2H), 2.09 (1H), 1.45 (s, 9H), 0.86–0.82 (6H);  **$^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )**  $\delta$  (ppm) 170.7, 170.5, 156.0, 136.4, 129.5, 128.8, 128.6, 128.3, 128.1, 127.1, 82.2, 67.1, 57.7, 56.3, 38.5, 31.5, 28.2, 18.8, 17.8; **MS (ESI)** :  $m/z$  = 455.1  $[\text{M}+\text{H}]^+$

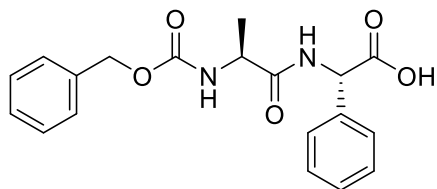
- Z-Phe-D-Val-OtBu [2142626-54-6]



Z-Phe-OH (311.9 mg, 1.042 mmol), H-D-Val-OtBu (270.8 mg, 1.563 mmol, 1.5 equiv.), Oxyma (117.7 mg, 1.250 mmol, 1.2 equiv.) and EtOAc (495  $\mu\text{L}$ ,  $\eta$  = 0.45  $\mu\text{L}/\text{mg}$ ) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (339.6 mg, 1.771 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 20 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 10 mL), 1N aqueous NaOH solution (2 x 10 mL) and brine (1 x 15 mL) and then dried over  $\text{MgSO}_4$ , filtered and concentrated under reduced pressure to afford 437 mg of the desired product as a yellow solid (92% yield).

**$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )**  $\delta$  (ppm) 7.39–7.14 (10H), 6.41 (d,  $J$  = 6.9 Hz, 1H), 5.42 (d,  $J$  = 7.4 Hz, 1H), 5.09 (s, 2H), 4.48 (br d,  $J$  = 7.0 Hz, 1H), 4.35 (dd,  $J$  = 8.5, 4.6 Hz, 1H), 3.08 (d,  $J$  = 6.6 Hz, 2H), 2.09 (m, 1H), 1.45 (s, 9H), 0.84 (t,  $J$  = 6.5 Hz, 6H);  **$^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )**  $\delta$  (ppm) 170.7, 170.5, 156.0, 136.4, 129.4, 128.8, 128.6, 128.3, 128.1, 127.1, 82.1, 67.1, 57.7, 56.3, 38.5, 31.5, 28.1, 18.8, 17.8; **MS (ESI)** :  $m/z$  = 455.2  $[\text{M}+\text{H}]^+$

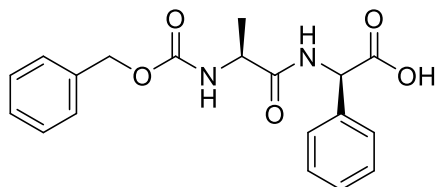
- Z-Ala-Phg-OH



In a 25 mL round-bottomed flask, Z-Ala-Phg-OtBu (419.8 mg, 1.018 mmol) in DCM (10 mL) was introduced. TFA (392.6  $\mu\text{L}$ , 5.089 mmol, 5 equiv.) was added and the reaction mixture was stirred at room temperature for 2h. The volatile compounds were evaporated under reduced pressure and the crude was dissolved in EtOAc (15 mL) and washed twice with 1N aqueous HCl (2 x 10 mL) and brine (1 x 10 mL). The organic phase was dried over  $\text{MgSO}_4$ , filtered and concentrated under reduced pressure to afford the desired product as a white solid ( $m$  = 329 mg, 91% yield).

**<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)** δ (ppm) 8.51 (d, *J* = 7.4 Hz, 1H), 7.48 (d, *J* = 7.8 Hz, 1H), 7.44–7.22 (10H), 5.32 (d, *J* = 7.4 Hz, 1H), 5.03–4.94 (2H), 4.2 (qt, *J* = 7.4 Hz, 1H), 1.24 (d, *J* = 7.1 Hz, 3H), **<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>)** δ (ppm) 172.4, 171.8, 155.7, 137.03, 136.99, 128.5, 128.4, 128.0, 127.8, 127.7, 127.6, 65.4, 56.2, 49.7, 18.2; **HRMS (ESI)**: calcd for C<sub>19</sub>H<sub>21</sub>N<sub>2</sub>O<sub>5</sub> = 357.1450 [M+H]<sup>+</sup>; found = 357.1453.

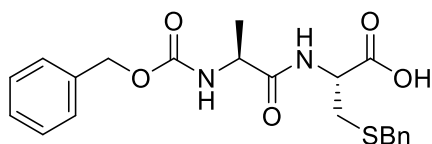
- Z-Ala-D-Phg-OH



In a 50 mL round-bottomed flask, Z-Ala-D-Phg-OtBu (971.6 mg, 1.284 mmol) in DCM (15 mL) was introduced. TFA (2.7 mL, 35.33 mmol, 15 equiv.) was added and the reaction mixture was stirred at room temperature for 1h. The volatile compounds were evaporated under reduced pressure and the crude was dissolved in EtOAc (20 mL). The organic phase was washed twice with brine (1 x 7 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford the desired product as a white solid (m = 816 mg, 97% yield).

**<sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 7.41–7.25 (10H), 5.43 (s, 1H), 5.11 (d, *J* = 13.0, 1H), 5.07 (d, *J* = 13.0, 1H), 4.26 (qt, *J* = 7.4 Hz, 1H), 1.31 (d, *J* = 7.1 Hz, 3H); **<sup>13</sup>C NMR (101 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 174.9, 173.3, 158.2, 138.2, 129.7, 129.5, 129.3, 129.0, 128.8, 128.5, 67.7, 57.9, 51.9, 18.3; **HRMS (ESI)**: calcd for C<sub>19</sub>H<sub>21</sub>N<sub>2</sub>O<sub>5</sub> = 357.1450 [M+H]<sup>+</sup>; found = 357.1451.

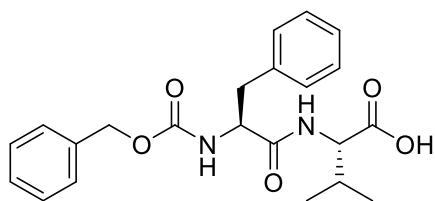
- Z-Ala-Cys(Bn)-OH [185454-03-9]



In a 50 mL round-bottomed flask, Z-Ala-Cys(Bn)-OtBu (775.5 mg, 1.641 mmol) in DCM (10 mL) was introduced. TFA (658.3 μL, 8.533 mmol, 5.2 equiv.) was added and the reaction mixture was stirred at room temperature for 2h. The volatile compounds were evaporated under reduced pressure and the crude was dissolved in EtOAc (15 mL) and washed twice with 1N aqueous HCl (2 x 10 mL) and brine (1 x 10 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford the desired product as a white solid (m = 597 mg, 87% yield).

**<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)** δ (ppm) 8.20 (d, *J* = 8.0 Hz, 1H), 7.46 (d, *J* = 7.9 Hz, 1H), 7.38–7.21 (10H), 5.02 (s, 2H), 4.50–4.40 (m, 1H), 4.18–4.10 (m, 1H), 3.78–3.72 (2H), 2.82–2.78 (m, 1H), 2.73–2.66 (m, 1H), 1.22 (d, *J* = 7.1 Hz, 3H), **<sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>)** δ (ppm) 172.7, 172.1, 155.7, 138.3, 137.1, 129.0, 128.9, 128.4, 127.84, 127.77, 126.9, 65.4, 52.0, 49.9, 35.4, 32.3, 18.3; **MS (ESI)**: *m/z* = 417.2 [M+H]<sup>+</sup>.

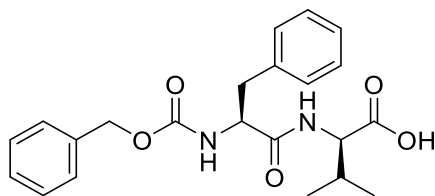
- Z-Phe-Val-OH<sup>3</sup> [13123-00-7]



In a 50 mL round-bottomed flask, Z-Phe-Val-OtBu (887.3 mg, 1.952 mmol) in DCM (15 mL) was introduced. TFA (2.3 mL, 29.280 mmol, 15.0 equiv.) was added portionwise on the reaction mixture that was stirred at room temperature for 3h. The volatile compounds were evaporated under reduced pressure and the crude was dissolved in EtOAc (15 mL) and washed with 1N aqueous HCl (2 x 10 mL) and brine (1 x 15 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford the desired product as a white solid (m = 706 mg, 91% yield).

**<sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 8.04 (d, *J* = 8.6 Hz, 1H), 7.33–7.17 (10H), 5.01 (s, 2H), 4.47 (dd, *J* = 9.5, 5.1 Hz, 1H), 4.37–4.30 (1H), 3.14 (dd, *J* = 13.9, 5.1 Hz, 1H), 2.85 (dd, *J* = 13.8, 9.7 Hz, 1H), 2.21–2.10 (m, 1H), 0.95 (d, *J* = 6.7 Hz, 6H); **<sup>13</sup>C NMR (101 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 174.4, 174.3, 158.2, 138.5, 138.2, 130.3, 129.41, 129.38, 128.9, 128.6, 127.7, 67.5, 58.9, 57.6, 39.0, 31.9, 19.5, 18.3; **MS (ESI):** *m/z* = 399.2 [M+H]<sup>+</sup>.

- Z-Phe-D-Val-OH [17460-97-8]

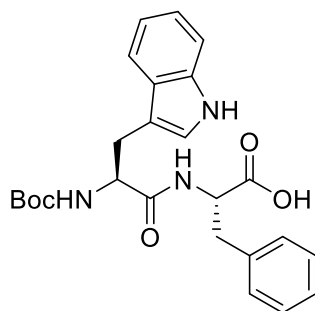


In a 25 mL round-bottomed flask, Z-Phe-D-Val-OtBu (412.0 mg, 0.924 mmol) in DCM (5 mL) was introduced. TFA (1.1 mL, 14 mmol, 15 equiv.) was added and the reaction mixture was stirred at room temperature for 1h. The volatile compounds were evaporated under reduced pressure and the crude was dissolved in EtOAc (10 mL). The organic phase was washed twice with 1N aqueous HCl solution (2 x 10 mL) and once with brine (1 x 10 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford the desired product as a white solid (m = 353 mg, 96% yield).

**<sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 8.04 (d, *J* = 8.6, 1H), 7.33–7.17 (10H), 5.00 (s, 2H), 4.48 (dd, *J* = 9.6, 5.1 Hz, 1H), 4.37–4.31 (1H), 3.14 (dd, *J* = 13.9, 5.1 Hz, 1H), 2.85 (dd, *J* = 13.8, 9.6 Hz, 1H), 2.21–2.11 (1H), 0.95 (d, *J* = 6.7 Hz, 6H); **<sup>13</sup>C NMR (101 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 174.4, 174.3, 158.2, 138.5, 138.2, 130.3, 129.41, 129.38, 128.9, 128.6, 127.7, 67.5, 58.9, 57.6, 39.0, 31.9, 19.5, 18.3; **MS (ESI):** *m/z* = 399.2 [M+H]<sup>+</sup>



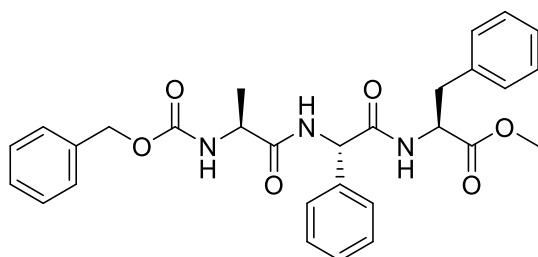
- Boc-Trp-Phe-OH [34337-45-6]



Boc-Trp-Phe-OMe (847.2 mg, 1.821 mmol),<sup>4</sup> LiOH·H<sub>2</sub>O (152.8 mg, 3.642 mmol, 2 equiv.) and 50/50 (v/v) H<sub>2</sub>O/MeOH (400  $\mu$ L,  $\eta$  = 0.40  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 40 minutes in the mixer mill operated at 25 Hz. Reaction mixture was recovered with deionised water and acidified up to pH 2. The resulting precipitate was filtered, washed twice with deionised water and dried under reduced pressure over P<sub>2</sub>O<sub>5</sub> to afford 701 mg of the desired product as a white solid (85% yield).

**<sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>)**  $\delta$  (ppm) 7.59 (d, *J* = 7.7 Hz, 1H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.27–6.98 (8H), 4.72–4.60 (1H), 4.39–4.30 (1H), 3.23–3.08 (2H), 3.06–2.94 (2H), 1.35 (s, 9H); **<sup>13</sup>C NMR (101 MHz, MeOD-*d*<sub>4</sub>)**  $\delta$  (ppm) 174.3, 138.1, 130.4, 129.4, 127.8, 124.6, 122.4, 119.8, 119.4, 112.3, 111.0, 80.7, 56.8, 54.9, 38.4, 29.1, 28.6; **MS (ESI):** *m/z* = 452.2 [M+H]<sup>+</sup>.

- Z-Ala-Phg-Phe-OMe

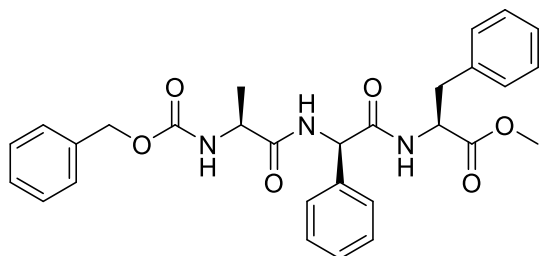


Z-Ala-Phg-OH (75.7 mg, 0.213 mmol), HCl·H-Phe-OMe (68.8 mg, 0.319 mmol, 1.5 equiv.), Oxyma (36.2 mg, 0.255 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (69.3 mg, 0.361 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 20 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 7 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 97.5 mg of the desired product as a yellow-orange solid (89% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)**  $\delta$  (ppm) 7.37–7.13 (14H), 7.08–7.02 (2H), 6.26 (br d, *J* = 6.6 Hz, 1H), 5.38–5.31 (2H), 5.08 (s, 2H), 4.80 (qd, *J* = 6.1 Hz, 1H), 4.37–4.25 (1H), 3.64 (s, 3H), 3.16 (dd, *J* = 13.8, 5.6 Hz, 1H), 3.05 (dd, *J* = 13.9, 6.1 Hz, 1H), 1.39 (d, *J* = 7.0 Hz, 3H); **<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>)**  $\delta$  (ppm) 171.7, 171.4, 169.3, 156.1, 137.0, 136.3, 135.6, 129.34, 129.26,

128.83, 128.78, 128.7, 128.3, 128.2, 127.43, 127.39, 67.2, 57.6, 53.7, 52.5, 50.7, 37.8, 18.8; **HRMS (ESI)**: calcd for  $C_{29}H_{32}N_3O_6$  = 518.2291  $[M+H]^+$ ; found = 518.2291. Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min,  $\lambda$  = 220.4 and 230.4 nm,  $t_r$  = 9.07 min (LLL) and  $t_r$  = 17.54 min (LDL), > 99% *de*.

- Z-Ala-D-Phg-Phe-OMe

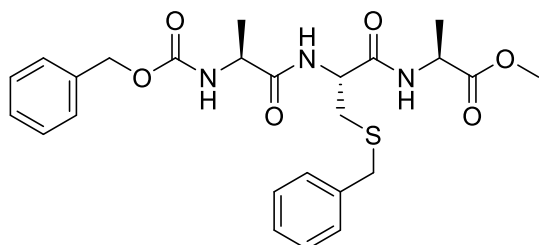


Z-Ala-D-Phg-OH (75.7 mg, 0.213 mmol), HCl·H-Phe-OMe (68.8 mg, 0.319 mmol, 1.5 equiv.), Oxyma (36.2 mg, 0.255 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (69.3 mg, 0.361 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 20 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 7 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous  $Na_2CO_3$  solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over  $MgSO_4$ , filtered and concentrated under reduced pressure to afford 101.2 mg of the desired product as a yellow powder (92% yield).

**$^1H$  NMR (500 MHz, DMSO- $d_6$ )**  $\delta$  (ppm) 8.92 (d,  $J$  = 7.5 Hz, 1H), 8.44 (d,  $J$  = 7.9 Hz, 1H), 7.53 (d,  $J$  = 7.5 Hz, 1H), 7.40–7.04 (15H), 5.55 (d,  $J$  = 8.0 Hz, 1H), 5.03 (s, 2H), 4.44 (m, 1H), 4.24–4.17 (1H), 3.63 (s, 3H), 3.03–2.96 (1H), 2.90–2.85 (1H), 1.18 (d,  $J$  = 7.0 Hz, 3H);  **$^{13}C$  NMR (125 MHz, DMSO- $d_6$ )**  $\delta$  (ppm) 172.1, 171.8, 169.6, 155.7, 138.7, 137.0, 136.8, 129.1, 128.4, 128.2, 128.1, 127.8, 127.7, 127.3, 126.5, 65.4, 55.5, 53.7, 52.0, 49.9, 36.6, 18.2; **HRMS (ESI)**: calcd for  $C_{29}H_{32}N_3O_6$  = 518.2291  $[M+H]^+$ ; found = 518.2288.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min,  $\lambda$  = 220.4 and 230.4 nm,  $t_r$  = 9.07 min (LLL) and  $t_r$  = 17.54 min (LDL), > 99% *de*.

- Z-Ala-Cys(Bn)-Ala-OMe



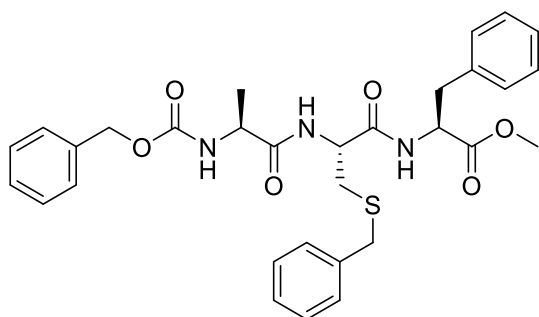
Z-Ala-Cys(Bn)-OH (101.4 mg, 0.244 mmol), HCl·H-Ala-OMe (51.0 mg, 0.365 mmol, 1.5 equiv.), Oxyma (41.5 mg, 0.292 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (79.5 mg, 0.415 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 30 minutes in the mixer mill operated at 25 Hz. The reaction mixture

was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 7 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 10 mL), 1N aqueous NaOH solution (2 x 10 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 119.4 mg of the desired product as a brown solid (98% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.39–7.18 (10H), 7.01 (d, *J* = 6.1 Hz, 1H), 6.92 (d, *J* = 5.6 Hz, 1H), 5.40 (d, *J* = 6.8 Hz, 1H), 5.11 (s, 2H), 4.59–4.47 (2H), 4.25 (br m, 1H), 3.76–3.70 (5H), 2.98–2.86 (1H), 2.79–2.67 (1H), 1.42–1.32 (6H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 172.9, 172.4, 169.7, 156.2, 138.2, 136.2, 129.2, 128.8, 128.7, 128.4, 128.2, 127.4, 67.3, 52.6, 52.3, 51.0, 48.5, 36.7, 33.6, 18.7, 18.1; **HRMS (ESI)**: calcd for C<sub>25</sub>H<sub>32</sub>N<sub>3</sub>O<sub>6</sub>S = 502.2012 [M+H]<sup>+</sup>; found = 502.2019.

Diastereomeric excess was determined by HPLC analysis (Chiralpak AS-H, heptane/iPrOH: 15/85), Flow rate = 1 mL/min, λ = 214 nm, t<sub>r</sub> = 17.50 min (LLL) and t<sub>r</sub> = 27.25 min (LDL), > **99% de**.

- Z-Ala-Cys(Bn)-Phe-OMe

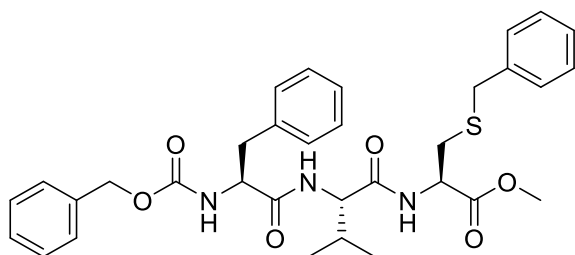


Z-Ala-Cys(Bn)-OH (91.3 mg, 0.219 mmol), HCl·H-Phe-OMe (70.9 mg, 0.329 mmol, 1.5 equiv.), Oxyma (37.4 mg, 0.263 mmol, 1.2 equiv.) and EtOAc (113 μL, η = 0.45 μL/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (71.4 mg, 0.372 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 20 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 10 mL), 1N aqueous NaOH solution (2 x 10 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 119.2 mg of the desired product as a yellow powder (94% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.33–7.12 (13H), 7.06–6.99 (2H), 6.75 (br d, *J* = 6.3 Hz, 1H), 6.67 (br d, *J* = 5.5 Hz, 1H), 5.18 (d, *J* = 6.3 Hz, 1H), 5.05 (d, *J* = 12.2 Hz, 1H), 5.01 (d, *J* = 12.3 Hz, 1H), 4.73 (qd, *J* = 6.4 Hz, 1H), 4.41–4.32 (1H), 4.14–4.07 (1H), 3.64 (s, 3H), 3.62 (s, 2H), 3.07 (dd, *J* = 13.9, 5.7 Hz, 1H), 2.99 (dd, *J* = 13.9, 6.4 Hz, 1H), 2.79 (br dd, *J* = 13.2, 4.5 Hz, 1H), 2.66–2.55 (1H), 1.26 (d, *J* = 7.0 Hz, 3H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 172.3, 171.5, 169.8, 138.1, 136.2, 135.8, 129.4, 129.1, 128.81, 128.77, 128.7, 128.4, 128.3, 127.4, 127.3, 67.3, 53.7, 52.5, 52.3, 50.8, 37.8, 36.7, 33.4, 18.6; **HRMS (ESI)**: calcd for C<sub>31</sub>H<sub>36</sub>N<sub>3</sub>O<sub>6</sub>S = 578.2325 [M+H]<sup>+</sup>; found = 578.2328.

Diastereomeric excess was determined by HPLC analysis (Chiralpak AS-H, heptane/iPrOH: 15/85), Flow rate = 1 mL/min, λ = 214nm, t<sub>r</sub> = 17.45 min (LLL) and t<sub>r</sub> = 24.45 min (LDL), > **99% de**.

- Z-Phe-Val-Cys(Bn)-OMe

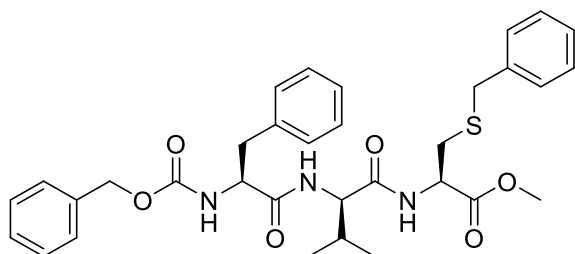


Z-Phe-Val-OH (83.6 mg, 0.210 mmol), HCl·H-Cys(Bn)-OMe (82.4 mg, 0.315 mmol, 1.5 equiv.), Oxyma (35.8 mg, 0.252 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (48.3 mg, 0.252 mmol, 1.2 equiv.) was added and the jar was closed and subjected to grinding for 30 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 10 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 10 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure. The resulting solid was triturated with Et<sub>2</sub>O to afford 124.2 mg of the desired product as a white powder (98% yield).

**<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)**  $\delta$  (ppm) 8.55 (d, *J* = 7.4 Hz, 1H), 7.95 (d, *J* = 9.0 Hz, 1H), 7.53 (d, *J* = 8.7 Hz, 1H), 7.37–7.13 (15H), 4.98–4.89 (2H), 4.49 (dd, *J* = 13.4, 7.7 Hz, 1H), 4.41–4.25 (2H), 3.76 (s, 2H), 3.62 (s, 3H), 3.02 (dd, *J* = 13.7, 3.2 Hz, 1H), 2.85–2.65 (3H), 2.05–1.94 (m, 1H), 0.93–0.80 (6H); **<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>)**  $\delta$  (ppm) 171.5, 171.1, 171.0, 155.9, 138.2, 138.1, 137.0, 129.2, 128.9, 128.4, 128.3, 128.0, 127.7, 127.4, 126.9, 126.2, 65.2, 57.3, 56.1, 52.0, 51.9, 37.3, 35.3, 31.9, 31.1, 19.1, 18.0; **HRMS (ESI)**: calcd for C<sub>33</sub>H<sub>40</sub>N<sub>3</sub>O<sub>6</sub>S = 606.2638 [M+H]<sup>+</sup>; found = 606.2645.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IF, heptane/ethanol: 50/50), Flow rate = 1 mL/min,  $\lambda$  = 220.4 and 230.4 nm, *t<sub>r</sub>* = 6.46 min (LLL) and *t<sub>r</sub>* = 10.05 min (LDL), > 99% *de*.

- Z-Phe-D-Val-Cys(Bn)-OMe



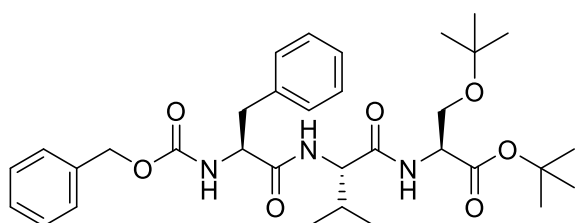
Z-Phe-D-Val-OH (77.4 mg, 0.194 mmol), HCl·H-Cys(Bn)-OMe (76.2 mg, 0.291 mmol, 1.5 equiv.), Oxyma (33.1 mg, 0.233 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (63.3 mg, 0.330 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 30 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with

EtOAc (2 x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), 1N aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 113.6 mg of the desired product as a yellow powder (97% yield).

**<sup>1</sup>H (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.39–7.07 (16H), 6.53 (d, *J* = 8.6 Hz, 1H), 5.63 (d, *J* = 7.1 Hz, 1H), 5.07 (d, *J* = 12.2 Hz, 1H), 5.02 (d, *J* = 12.5 Hz, 1H), 4.75 (m, 1H), 4.52–4.35 (2H), 3.73–3.60 (5H), 3.13–3.01 (2H), 2.88 (dd, *J* = 13.8, 5.2 Hz, 1H), 2.76 (dd, *J* = 13.8, 6.4 Hz, 1H), 2.11–1.97 (m, 1H), 0.80–0.69 (6H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 171.7, 171.2, 170.7, 156.2, 137.7, 136.4, 136.2, 129.4, 129.1, 128.9, 128.7, 128.6, 128.3, 128.2, 127.3, 127.2, 67.2, 58.2, 56.9, 52.7, 51.7, 38.6, 36.4, 33.3, 30.7, 19.1, 17.7; **HRMS (ESI)**: calcd for C<sub>33</sub>H<sub>40</sub>N<sub>3</sub>O<sub>6</sub>S = 606.2638 [M+H]<sup>+</sup>; found = 606.2649.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IF, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 220.4 and 230.4 nm, t<sub>r</sub> = 6.46 min (LLL) and t<sub>r</sub> = 10.05 min (LDL), > 99% *de*.

- Z-Phe-Val-Ser(*t*Bu)-O*t*Bu

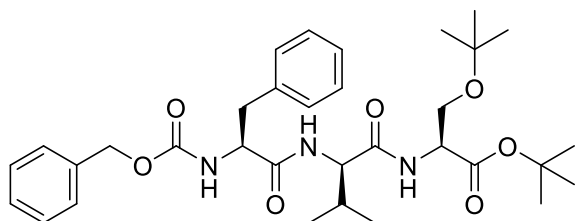


Z-Phe-Val-OH (84.4 mg, 0.212 mmol), HCl·H-Ser(*t*Bu)-O*t*Bu (80.7 mg, 0.318 mmol, 1.5 equiv.), Oxyma (36.1 mg, 0.254 mmol, 1.2 equiv.) and EtOAc (113 μL, η = 0.45 μL/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (48.8 mg, 0.254 mmol, 1.2 equiv.) was added and the jar was closed and subjected to grinding for 30 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 10 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 10 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure. The resulting solid was triturated with Et<sub>2</sub>O to afford 123.4 mg of the desired product as a yellow solid (97% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.35–7.12 (10H), 6.64 (br d, *J* = 7.6 Hz, 1H), 6.49 (br d, *J* = 8.2 Hz, 1H), 5.40 (br d, *J* = 8.0 Hz, 1H), 5.07 (s, 2H), 4.61–4.53 (1H), 4.51–4.46 (1H), 4.30 (dd, *J* = 8.4, 6.0 Hz, 1H), 3.79 (dd, *J* = 8.7, 2.8 Hz, 1H), 3.54–3.45 (1H), 3.08 (d, *J* = 6.4 Hz, 2H), 2.12–2.03 (m, 1H), 1.46 (s, 9H), 1.15 (s, 9H), 0.92 (d, *J* = 6.8 Hz, 3H), 0.88 (d, *J* = 6.8 Hz, 3H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 170.9, 170.1, 169.2, 156.1, 136.4, 129.5, 128.8, 128.6, 128.3, 128.2, 127.1, 82.1, 73.3, 67.1, 62.3, 58.5, 56.2, 53.3, 38.4, 31.7, 28.2, 27.5, 19.1, 18.0; **HRMS (ESI)**: calcd for C<sub>33</sub>H<sub>48</sub>N<sub>3</sub>O<sub>7</sub> = 598.3492 [M+H]<sup>+</sup>; found = 598.3501

Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 220.4 and 230.4 nm, t<sub>r</sub> = 6.09 min (LLL) and t<sub>r</sub> = 9.81 min (LDL), > 99% *de*.

- Z-Phe-D-Val-Ser(*t*Bu)-O*t*Bu

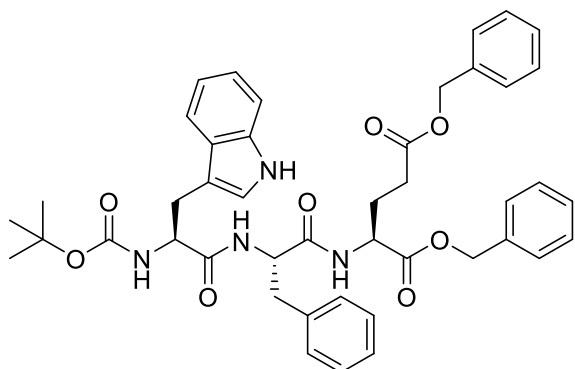


Z-Phe-D-Val-OH (78.1 mg, 0.196 mmol), HCl·H-Ser(*t*Bu)-O*t*Bu (74.6 mg, 0.294 mmol, 1.5 equiv.), Oxyma (33.4 mg, 0.235 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (63.9 mg, 0.333 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 20 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 114.1 mg of the desired product as a yellow solid (97% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)**  $\delta$  (ppm) 7.37–7.16 (10H), 6.69 (br s, 1H), 6.46 (br d, *J* = 8.4 Hz, 1H), 5.51 (br d, *J* = 6.5 Hz, 1H), 5.09 (d, *J* = 12.3 Hz, 1H), 5.05 (d, *J* = 12.3 Hz, 1H), 4.60–4.53 (1H), 4.52–4.41 (1H), 4.35 (dd, *J* = 8.1, 6.2 Hz, 1H), 3.73 (dd, *J* = 8.6, 2.8 Hz, 1H), 3.53 (dd, *J* = 8.6, 2.9 Hz, 1H), 3.15–2.99 (2H), 1.96 (m, 1H), 1.44 (s, 9H), 1.11 (s, 9H), 0.78 (t, *J* = 6.4 Hz, 6H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**  $\delta$  (ppm) 170.7, 169.9, 169.2, 156.0, 136.6, 129.4, 128.9, 128.6, 128.24, 128.21, 127.1, 82.2, 73.2, 67.1, 62.1, 58.2, 56.7, 53.5, 39.0, 31.4, 28.1, 27.5, 18.8, 18.2; **HRMS (ESI)**: calcd for C<sub>33</sub>H<sub>48</sub>N<sub>3</sub>O<sub>7</sub> = 598.3492 [M+H]<sup>+</sup>; found = 598.3500.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min,  $\lambda$  = 220.4 and 230.4 nm, *t<sub>r</sub>* = 6.09 min (LLL) and *t<sub>r</sub>* = 9.81 min (LDL), > 99% *de*.

- Boc-Trp-Phe-Glu(Bn)-OBn



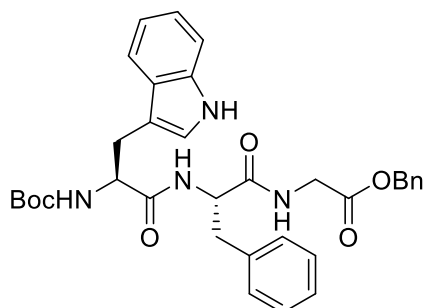
Z-Trp-Phe-OH (66.5 mg, 0.147 mmol), TsOH·H-Glu(Bn)-OBn (110.4 mg, 0.221 mmol, 1.5 equiv.), Oxyma (25.1 mg, 0.177 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (48.0 mg, 0.250 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 15 minutes in the mixer mill operated at 25 Hz. The reaction mixture

was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 7 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 102.6 mg of the desired product as a yellow powder (92% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 8.28 (s, 1H), 7.66 (d, *J* = 7.9 Hz, 1H), 7.41–7.27 (11H), 7.22–7.06 (5H), 6.96 (s, 1H), 6.91–6.82 (2H), 6.51 (br d, *J* = 6.8 Hz, 1H), 6.07 (d, *J* = 7.8 Hz, 1H), 5.16–5.01 (5H), 4.61–4.48 (2H), 4.42–4.29 (1H), 3.32–3.22 (1H), 3.10 (dd, *J* = 14.5, 7.4 Hz, 1H), 3.03–2.94 (1H), 2.78–2.63 (1H), 2.35–2.08 (3H), 1.94–1.80 (1H), 1.35 (s, 9H); **<sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)** δ (ppm) 172.8, 171.6, 171.0, 170.5, 155.7, 136.4, 136.1, 135.8, 135.3, 129.3, 128.8, 128.6, 128.5, 128.4, 127.4, 127.1, 123.3, 122.5, 120.0, 119.1, 111.5, 110.4, 80.2, 67.4, 66.7, 55.2, 54.1, 51.8, 37.4, 30.1, 28.3, 27.2; **HRMS (ESI)**: calcd for C<sub>44</sub>H<sub>49</sub>N<sub>4</sub>O<sub>8</sub> = 761.3550 [M+H]<sup>+</sup>; found = 761.3564.

Diastereomeric excess was determined by HPLC analysis (Chiralpak ID, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 205.4 and 220.4 nm, t<sub>r</sub> = 7.20 min (LLL) and t<sub>r</sub> = 10.93 min (LDL), > 99% *de*.

- Boc-Trp-Phe-Gly-OBn

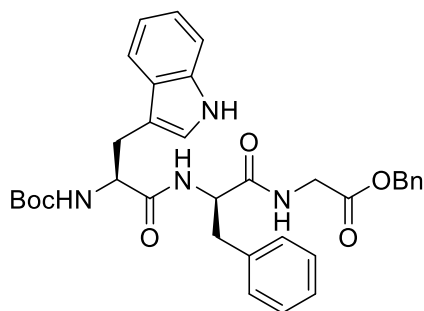


Boc-Trp-Phe-OH (77.6 mg, 0.172 mmol), TsOH·H-Gly-OBn (87.0 mg, 0.258 mmol, 1.5 equiv.), Oxyma (29.3 mg, 0.206 mmol, 1.2 equiv.) and EtOAc (113 μL, η = 0.45 μL/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (56.0 mg, 0.292 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 10 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 7 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 86.4 mg of the desired product as a white powder (84% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 8.05 (s, 1H), 7.62 (d, *J* = 7.8 Hz, 1H), 7.41–7.09 (11H), 6.97–6.84 (3H), 6.45 (br s, 1H), 6.06 (d, *J* = 6.1 Hz, 1H), 5.18–5.12 (2H), 5.00 (br s, 1H), 4.67–4.61 (1H), 4.32 (qd, *J* = 6.2 Hz, 1H), 3.96 (dd, *J* = 17.8, 5.8 Hz, 1H), 3.75 (dd, *J* = 17.8, 4.7 Hz, 1H), 3.22 (dd, *J* = 14.1, 5.1 Hz, 1H), 3.11 (dd, *J* = 14.4, 6.8 Hz, 1H), 3.05–2.95 (1H), 2.81 (dd, *J* = 13.8, 6.5 Hz, 1H), 1.34 (s, 9H); **<sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)** δ (ppm) 171.7, 171.0, 169.3, 136.33, 136.30, 135.5, 129.4, 128.79, 128.75, 128.7, 128.5, 127.4, 127.0, 123.3, 122.7, 120.1, 119.0, 111.4, 110.3, 81.0, 67.2, 55.7, 53.6, 41.4, 37.2, 28.3; **HRMS (ESI)**: calcd for C<sub>34</sub>H<sub>39</sub>N<sub>4</sub>O<sub>6</sub> = 599.2870 [M+H]<sup>+</sup>; found = 599.2888

Diastereomeric excess was determined by HPLC analysis (Chiralpak IA, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 205.4 and 220.4 nm, t<sub>r</sub> = 10.14 min (LL) and t<sub>r</sub> = 7.40 min (LD), > 99% *de*.

- Boc-Trp-D-Phe-Gly-OBn

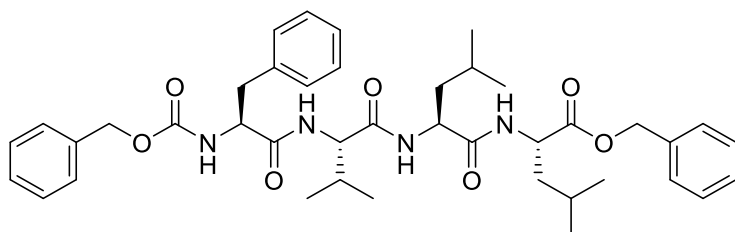


Boc-Trp-D-Phe-OH (77.6 mg, 0.172 mmol),<sup>5</sup> TsOH·H-Gly-OBn (87.0 mg, 0.258 mmol, 1.5 equiv.), Oxyma (29.3 mg, 0.206 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (56.0 mg, 0.292 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 10 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 7 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 97.3 mg of the desired product as a yellow powder (95% yield).

**<sup>1</sup>H (400 MHz, CDCl<sub>3</sub>)**  $\delta$  (ppm) 8.06 (br s, 1H), 7.59 (d,  $J$  = 7.8 Hz, 1H), 7.41–7.04 (11H), 6.96–6.92 (1H), 6.89–6.76 (3H), 6.05 (br d,  $J$  = 6.4 Hz, 1H), 5.16–5.06 (3H), 4.68 (m, 1H), 4.27–4.18 (1H), 4.02 (dd,  $J$  = 17.8, 4.4 Hz, 1H), 3.75 (dd,  $J$  = 17.9, 5.0 Hz, 1H), 3.20 (dd,  $J$  = 14.1, 5.8 Hz, 1H), 3.11 (dd,  $J$  = 14.1, 7.8 Hz, 1H), 3.03 (dd,  $J$  = 13.6, 5.0 Hz, 1H), 2.69–2.58 (1H), 1.37 (s, 9H); **<sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)**  $\delta$  (ppm) 172.2, 171.0, 169.4, 136.3, 136.1, 135.5, 129.5, 128.8, 128.6, 128.5, 127.4, 127.1, 123.4, 122.6, 120.0, 118.8, 111.5, 110.2, 80.6, 67.1, 56.2, 53.7, 41.4, 36.9, 28.4, 27.8; **HRMS (ESI)**: calcd for C<sub>34</sub>H<sub>39</sub>N<sub>4</sub>O<sub>6</sub> = 599.2870 [M+H]<sup>+</sup>; found = 599.2879.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IA, heptane/ethanol: 50/50), Flow rate = 1 mL/min,  $\lambda$  = 205.4 and 220.4 nm,  $t_r$  = 10.14 min (LL) and  $t_r$  = 7.40 min (LD), > **99% de**.

- Z-Phe-Val-Leu<sub>2</sub>-OBn



Z-Phe-Val-OH (78.7 mg, 0.198 mmol), HCl·H-Leu<sub>2</sub>-OBn (73.2 mg, 0.198 mmol, 1.0 equiv.),<sup>6</sup> Oxyma (33.7 mg, 0.237 mmol, 1.2 equiv.) and EtOAc (113  $\mu$ L,  $\eta$  = 0.45  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (64.4 mg, 0.336 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 25 minutes in the mixer mill operated at 25 Hz. The reaction mixture

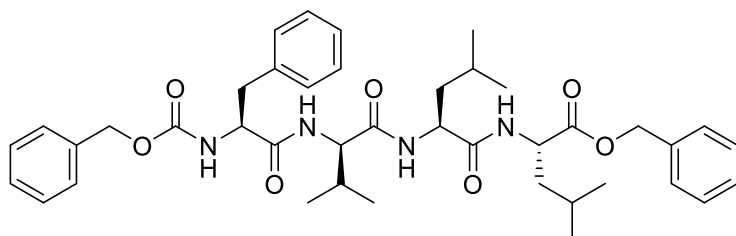


was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 134.2 mg of the desired product as an orange solid (95% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.85 (br s, 1H), 7.65 (br s, 1H), 7.39–7.05 (16H), 6.02 (br d, *J* = 6.0 Hz, 1H), 5.17 (d, *J* = 12.4 Hz, 1H), 5.12–5.00 (2H), 4.97–4.84 (2H), 4.79–4.64 (2H), 4.55–4.44 (1H), 3.07–2.92 (2H), 2.09–2.00 (m, 1H), 1.78–1.69 (m, 1H), 1.68–1.48 (5H), 0.95–0.75 (18H); **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ (ppm) 172.8, 172.0, 171.6, 171.2, 156.3, 136.6, 136.5, 135.7, 129.5, 128.6, 128.5, 128.4, 128.3, 128.1, 128.0, 126.9, 67.1, 66.9, 58.6, 56.0, 51.7, 50.9, 41.4, 41.0, 39.3, 31.7, 25.0, 24.9, 23.1, 22.8, 22.5, 21.9, 19.2, 18.5; **HRMS (ESI)**: calcd for C<sub>41</sub>H<sub>55</sub>N<sub>4</sub>O<sub>7</sub> = 715.4071 [M+H]<sup>+</sup>; found = 715.4076.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 205.4 and 220.4 nm, t<sub>r</sub> = 5.18 min (LLLL) and t<sub>r</sub> = 12.60 min (LDLL), > 98% **de**.

- Z-Phe-D-Val-Leu<sub>2</sub>-OBn

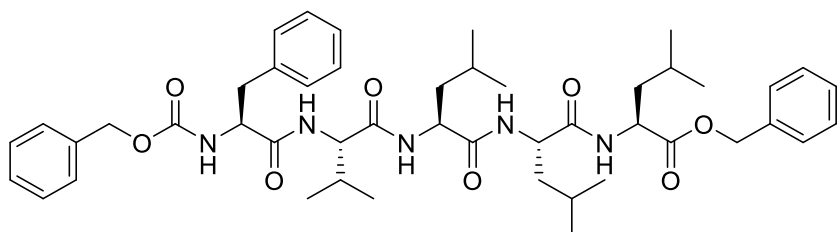


Z-Phe-D-Val-OH (78.7 mg, 0.198 mmol), HCl·H-Leu<sub>2</sub>-OBn (73.2 mg, 0.198 mmol, 1.0 equiv.),<sup>6</sup> Oxyma (33.7 mg, 0.237 mmol, 1.2 equiv.) and EtOAc (113 μL, η = 0.45 μL/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (64.4 mg, 0.336 mmol, 1.7 equiv.) was added and the jar was closed and subjected to grinding for 25 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 128.6 mg of the desired product as a yellow-orange solid (91% yield).

**<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ (ppm) 7.39–7.14 (15H), 6.81 (br d, *J* = 8.2 Hz, 1H), 6.59 (br d, *J* = 7.8 Hz, 1H), 6.54 (br d, *J* = 8.3 Hz, 1H), 5.65 (d, *J* = 7.2 Hz, 1H), 5.15–4.98 (4H), 4.70–4.61 (1H), 4.51–4.36 (2H), 4.13 (dd, *J* = 8.0, 6.5 Hz, 1H), 3.10 (dd, *J* = 13.7, 7.8 Hz, 1H), 2.99 (dd, *J* = 13.5, 6.9 Hz, 1H), 2.17–2.04 (m, 1H), 1.83–1.69 (2H), 1.66–1.47 (4H), 0.93–0.83 (12H), 0.78–0.68 (6H); **<sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)** δ (ppm) 173.2, 171.9, 171.8, 170.9, 156.5, 136.4, 136.0, 135.4, 129.3, 128.9, 128.72, 128.69, 128.5, 128.4, 128.3, 128.2, 127.2, 67.3, 67.2, 59.2, 56.9, 52.4, 50.8, 41.5, 40.8, 38.2, 24.92, 24.89, 23.1, 22.9, 22.0, 21.9, 19.2, 17.8; **HRMS (ESI)**: calcd for C<sub>41</sub>H<sub>55</sub>N<sub>4</sub>O<sub>7</sub> = 715.4077 [M+H]<sup>+</sup>; found = 715.4077.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 205.4 and 220.4 nm, t<sub>r</sub> = 5.18 min (LLLL) and t<sub>r</sub> = 12.60 min (LDLL), > 98% **de**.

- Z-Phe-Val-Leu<sub>3</sub>-OBn

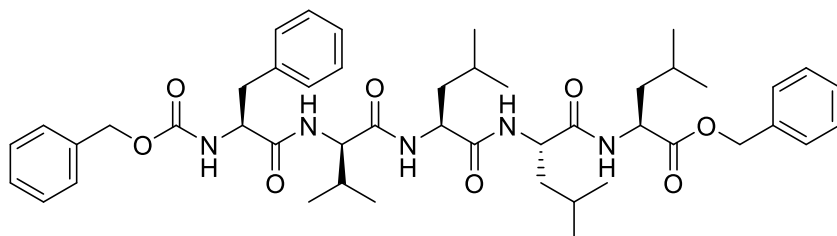


Z-Phe-Val-OH (72.2 mg, 0.181 mmol), HCl·H-Leu<sub>3</sub>-OBn (87.7 mg, 0.181 mmol, 1.0 equiv.),<sup>6</sup> Oxyma (30.9 mg, 0.218 mmol, 1.2 equiv.) and EtOAc (226  $\mu$ L,  $\eta$  = 0.9  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (76.5 mg, 0.399 mmol, 2.2 equiv.) was added and the jar was closed and subjected to grinding for 30 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2 x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 139.7 mg of the desired product as a yellow-orange powder (93% yield).

**<sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>, 60°C)**  $\delta$  (ppm) 8.09 (d,  $J$  = 7.7 Hz, 1H), 7.92 (d,  $J$  = 8.2 Hz, 1H), 7.80–7.73 (2H), 7.41–7.13 (16H), 5.11 (d,  $J$  = 12.6 Hz, 1H), 5.08 (d,  $J$  = 12.7 Hz, 1H), 4.95 (s, 2H), 4.40–4.29 (4H), 4.21 (dd,  $J$  = 8.8, 6.7 Hz, 1H), 3.00 (dd,  $J$  = 14.1, 3.2 Hz, 1H), 2.81–2.72 (1H), 2.08–1.92 (m, 1H), 1.69–1.36 (9H), 0.94–0.74 (24H); **<sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>, 60°C)**  $\delta$  (ppm) 171.9, 171.7, 171.24, 171.19, 170.4, 155.7, 137.9, 136.9, 135.8, 129.0, 128.2, 128.1, 127.8, 127.6, 127.5, 127.2, 126.0, 65.8, 65.1, 57.5, 56.0, 50.9, 50.6, 50.2, 40.9, 40.7, 37.2, 30.5, 28.8, 24.0, 23.8, 22.8, 22.7, 22.5, 21.6, 21.5, 21.1, 18.9, 17.8; **HRMS (ESI)**: calcd for C<sub>47</sub>H<sub>66</sub>N<sub>5</sub>O<sub>8</sub> = 828.4911 [M+H]<sup>+</sup>; found = 828.4886.

Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min,  $\lambda$  = 205.4 and 220.4 nm,  $t_r$  = 6.18 min (LLLLL) and  $t_r$  = 7.27 min (LDLLL), > 99% *de*.

- Z-Phe-D-Val-Leu<sub>3</sub>-OBn



Z-Phe-D-Val-OH (72.2 mg, 0.181 mmol), HCl·H-Leu<sub>3</sub>-OBn (87.7 mg, 0.181 mmol, 1.0 equiv.),<sup>6</sup> Oxyma (30.9 mg, 0.218 mmol, 1.2 equiv.) and EtOAc (226  $\mu$ L,  $\eta$  = 0.9  $\mu$ L/mg) were introduced in a 15 mL PTFE grinding jar with one stainless steel ball (10 mm diameter). The jar was closed and subjected to grinding for 1 minute in the mixer mill operated at 25 Hz. Then, EDC·HCl (76.5 mg, 0.399 mmol, 2.2 equiv.) was added and the jar was closed and subjected to grinding for 30 minutes in the mixer mill operated at 25 Hz. The reaction mixture was recovered with deionized water and EtOAc. The aqueous phase was extracted with EtOAc (2

x 5 mL). The combined organic phase was washed with 1N aqueous HCl solution (2 x 7 mL), saturated aqueous Na<sub>2</sub>CO<sub>3</sub> solution (2 x 7 mL) and brine (1 x 10 mL) and then dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford 136.6 mg of the desired product as a yellow-orange powder (91% yield).

**<sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 7.37–7.17 (15H), 5.16–4.97 (4H), 4.51–4.45 (1H), 4.45–4.39 (2H), 4.39–4.32 (1H), 3.94 (d, *J* = 7.0 Hz, 1H), 3.05–2.94 (2H), 2.03–1.96 (m, 1H), 1.74–1.49 (9H), 0.96–0.79 (19H), 0.73–0.67 (5H); **<sup>13</sup>C NMR (125 MHz, MeOD-*d*<sub>4</sub>)** δ (ppm) 174.63, 174.57, 174.2, 174.1, 173.8, 158.2, 138.1, 137.2, 130.4, 129.6, 129.5, 129.4, 129.3, 129.0, 128.8, 127.9, 67.9, 67.6, 61.0, 58.0, 53.4, 52.8, 52.3, 41.7, 41.3, 41.2, 39.2, 30.5, 25.81, 25.76, 23.54, 23.52, 23.3, 21.94, 21.88, 21.5, 19.6, 18.6; **HRMS (ESI)**: calcd for C<sub>47</sub>H<sub>66</sub>N<sub>5</sub>O<sub>8</sub> = 828.4911 [M+H]<sup>+</sup>; found = 828.4929.

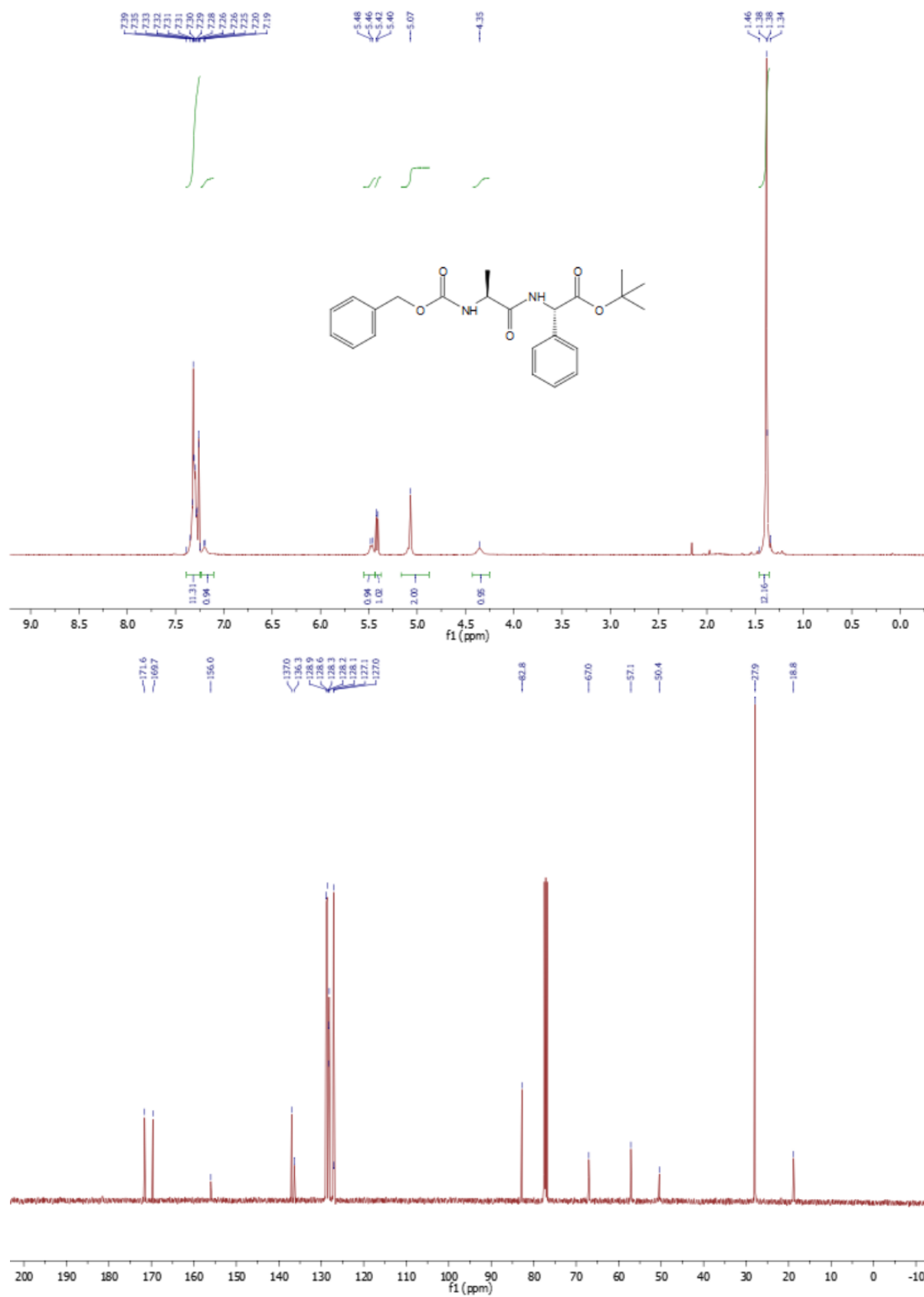
Diastereomeric excess was determined by HPLC analysis (Chiralpak IE, heptane/ethanol: 50/50), Flow rate = 1 mL/min, λ = 205.4 and 220.4 nm, t<sub>r</sub> = 6.18 min (LLLLL) and t<sub>r</sub> = 7.27 min (LDLLL), **99% de**.

# <sup>1</sup>H and <sup>13</sup>C NMR spectra

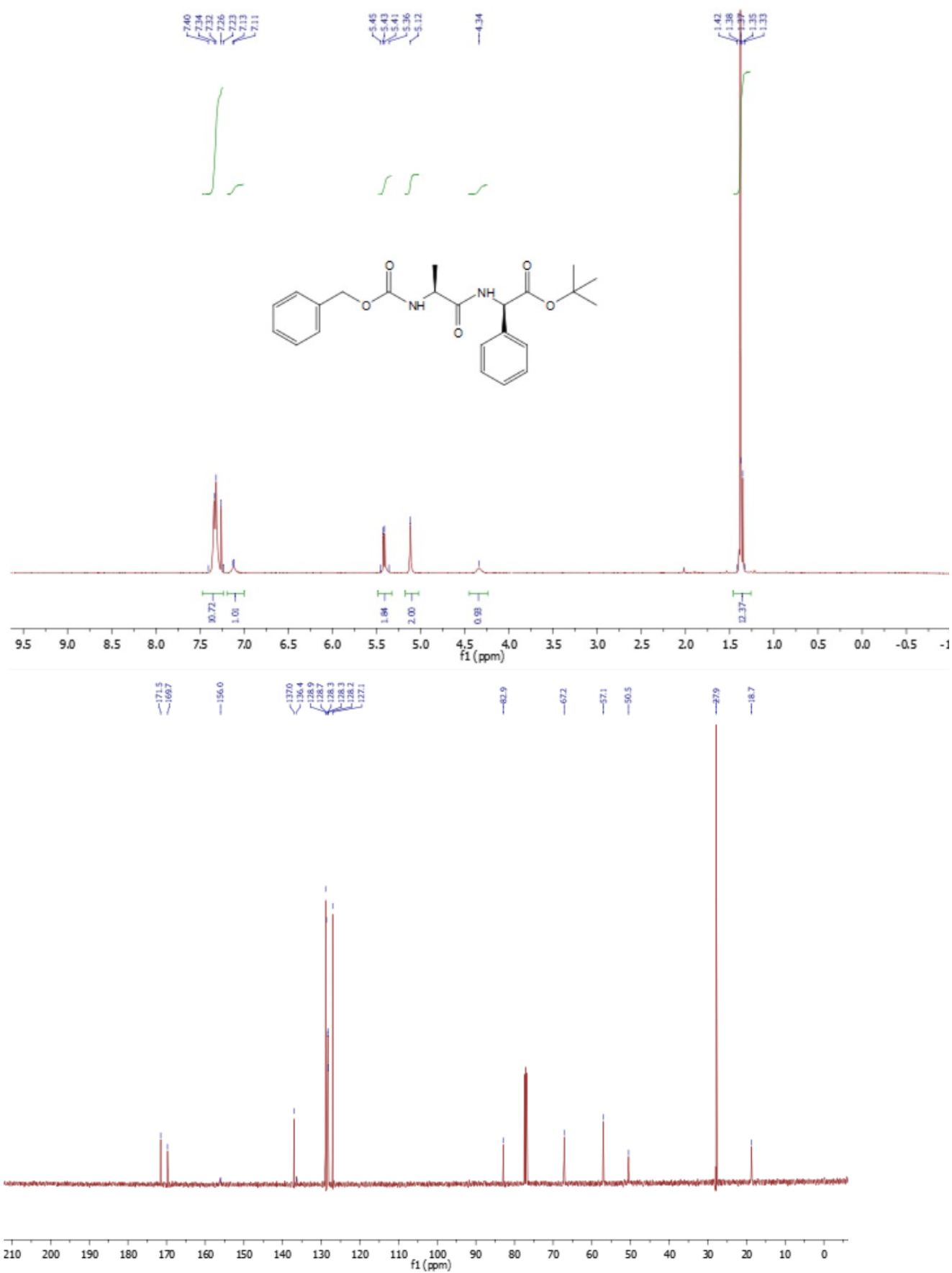
- Z-Ala-Phg-Ile-OMe



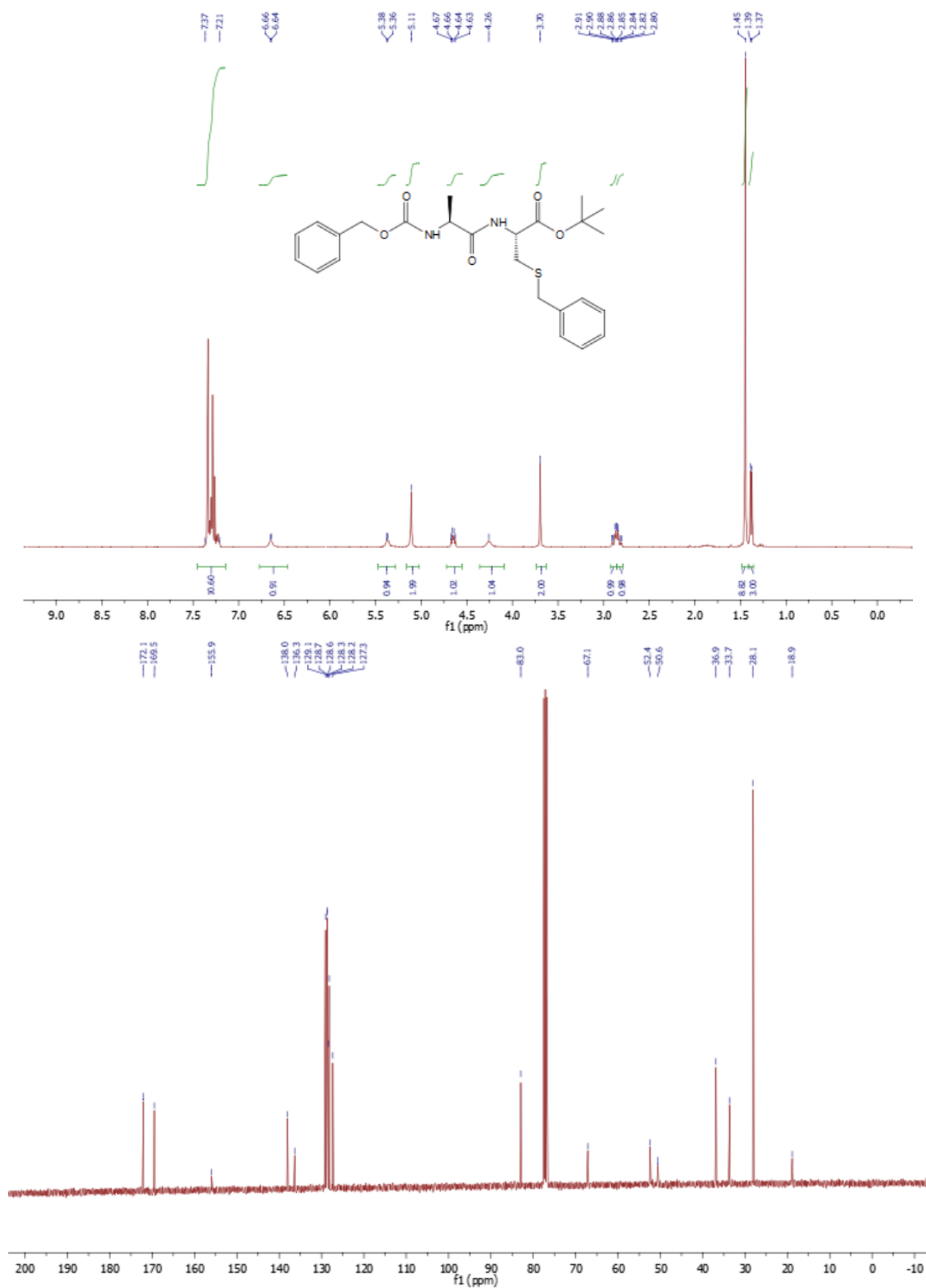
- Z-Ala-Phg-OfBu



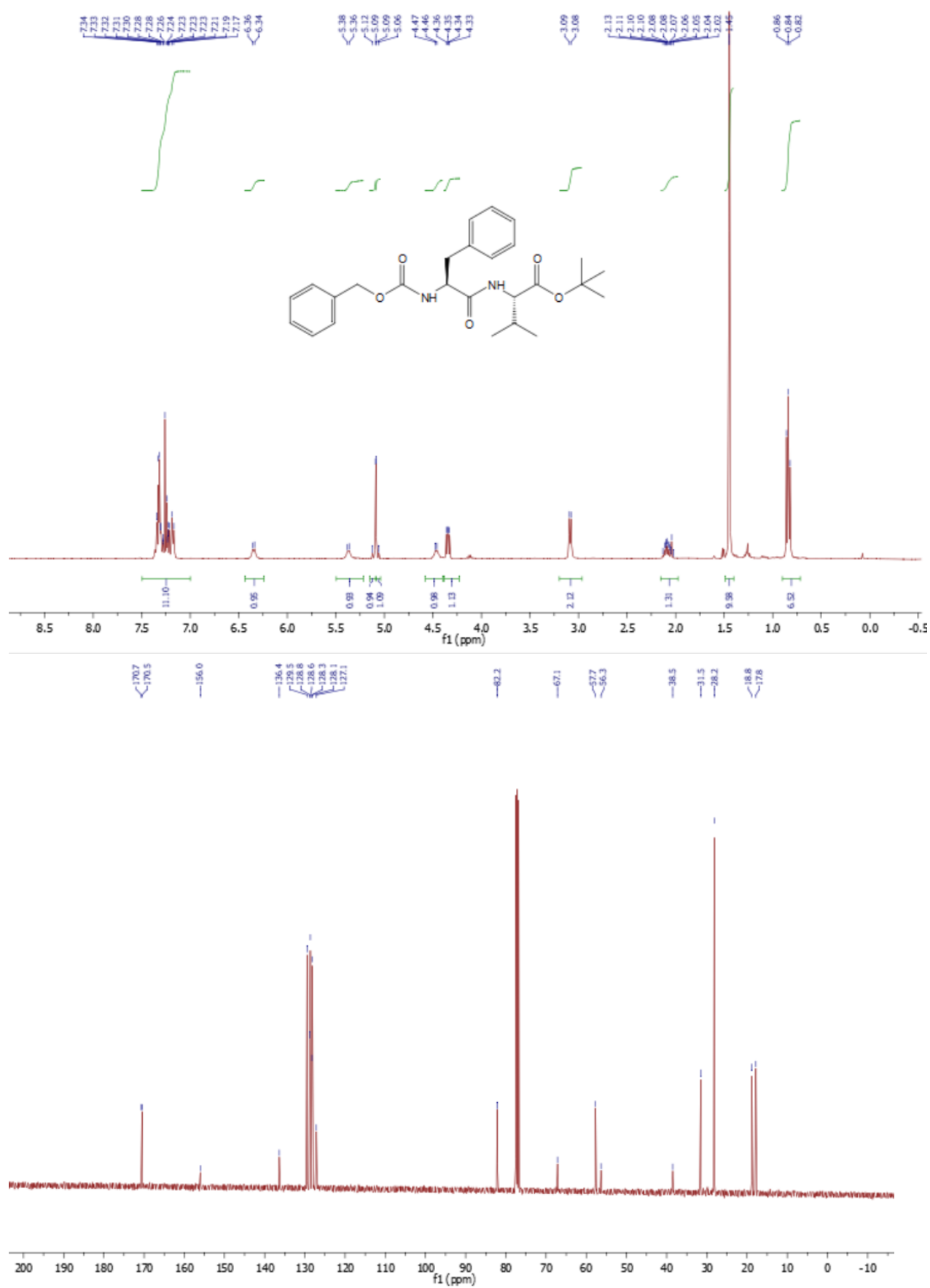
- Z-Ala-D-Phg-OtBu



- Z-Ala-Cys(Bn)-OtBu

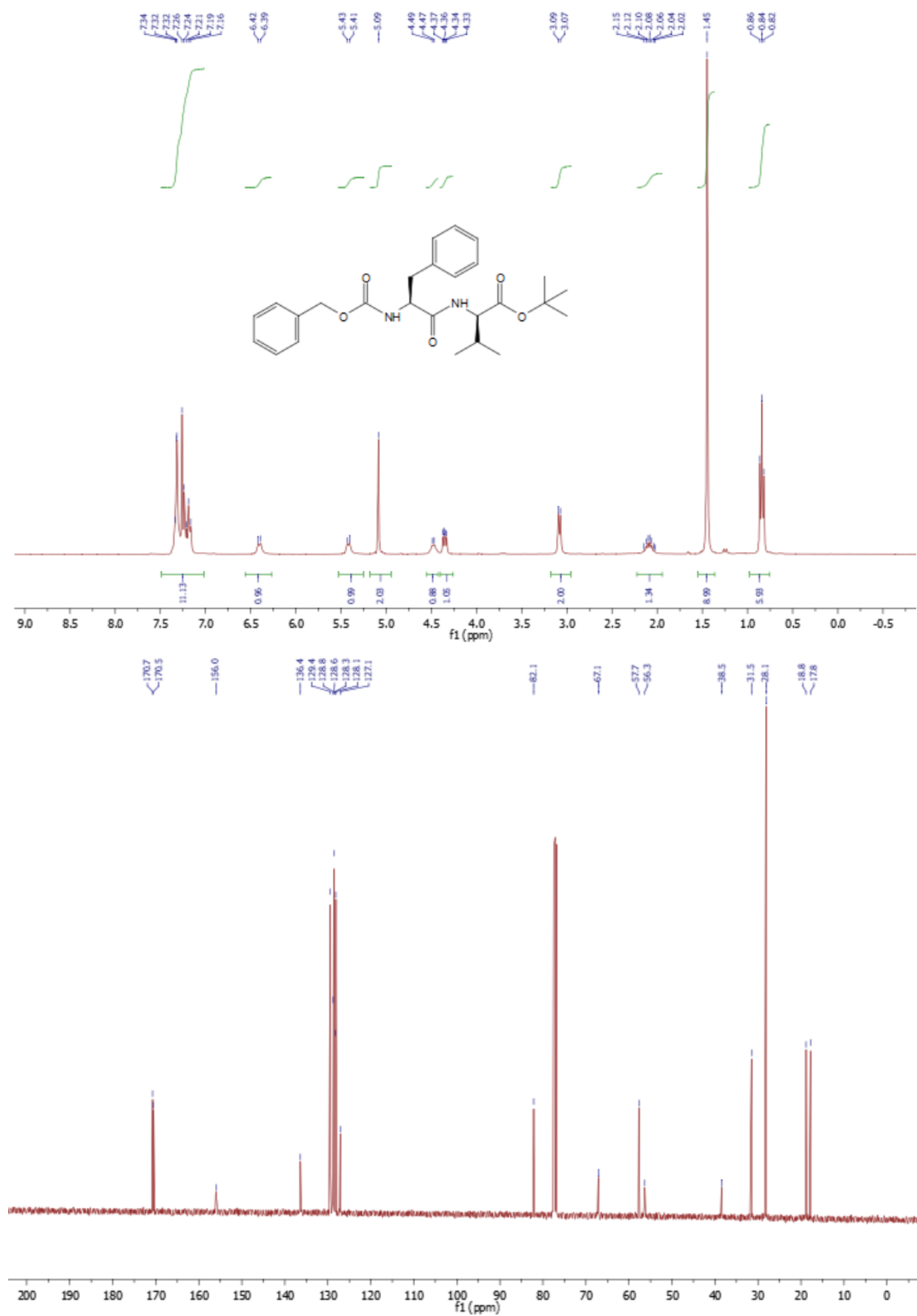


- Z-Phe-Val-OtBu

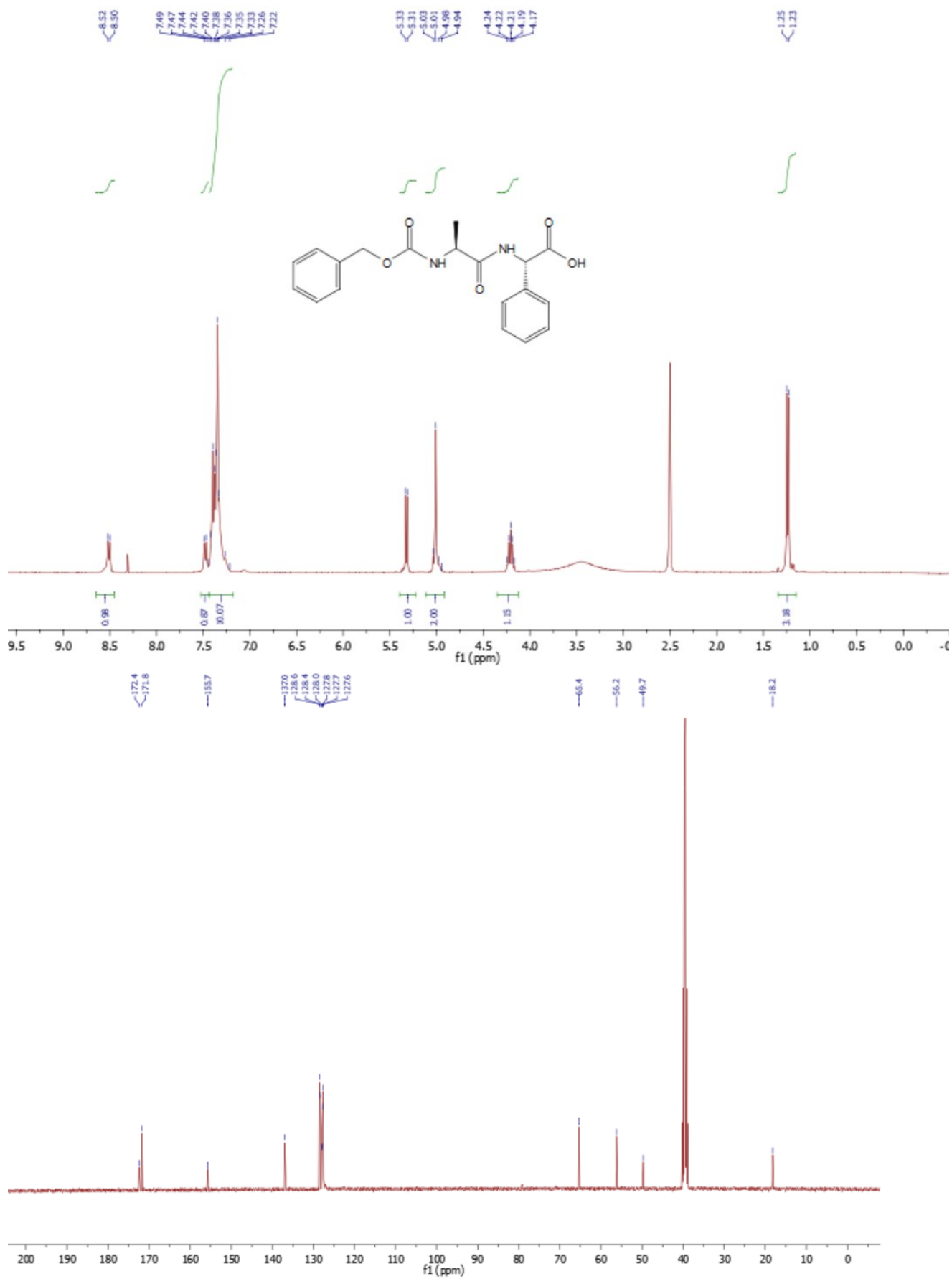




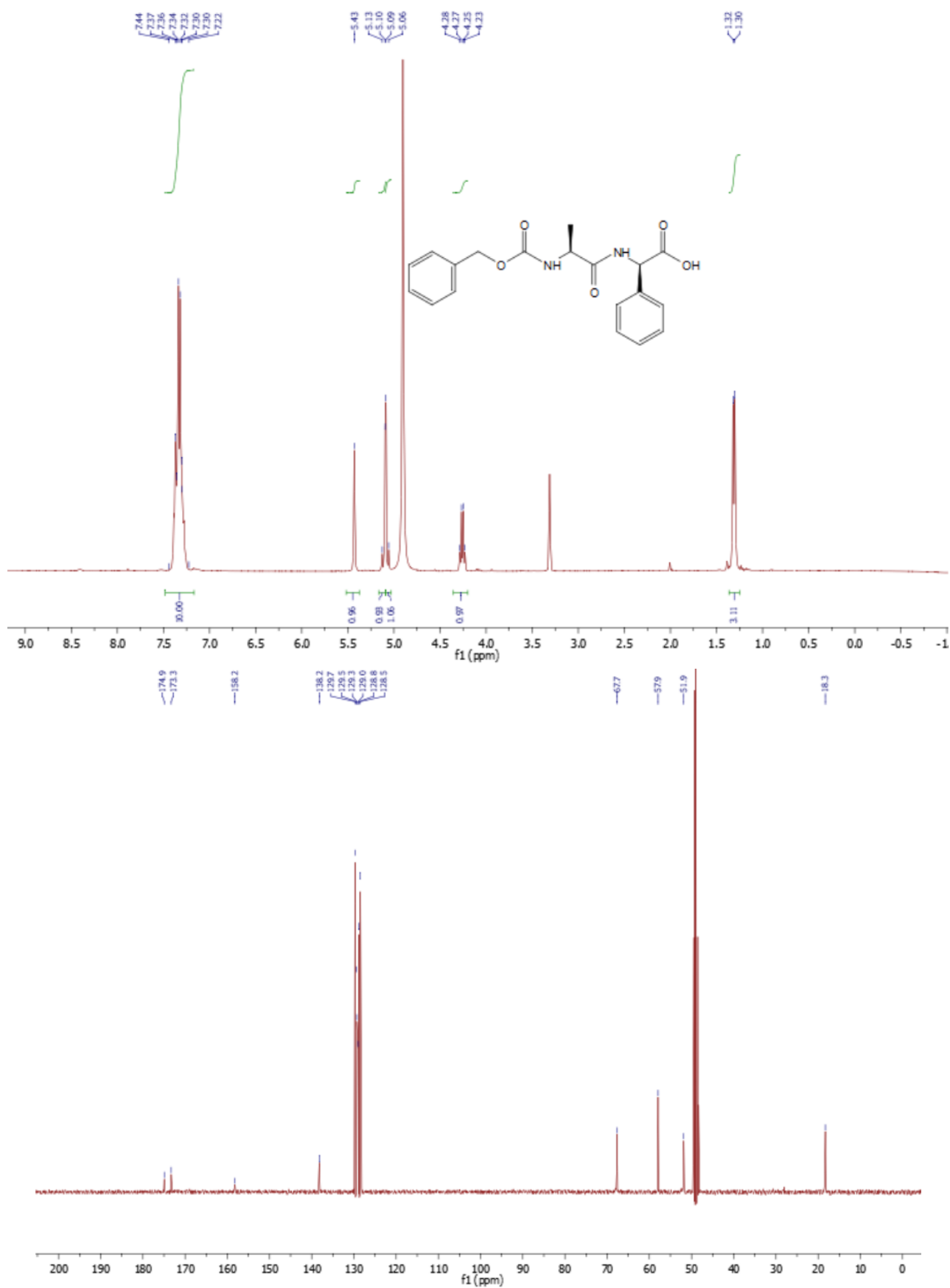
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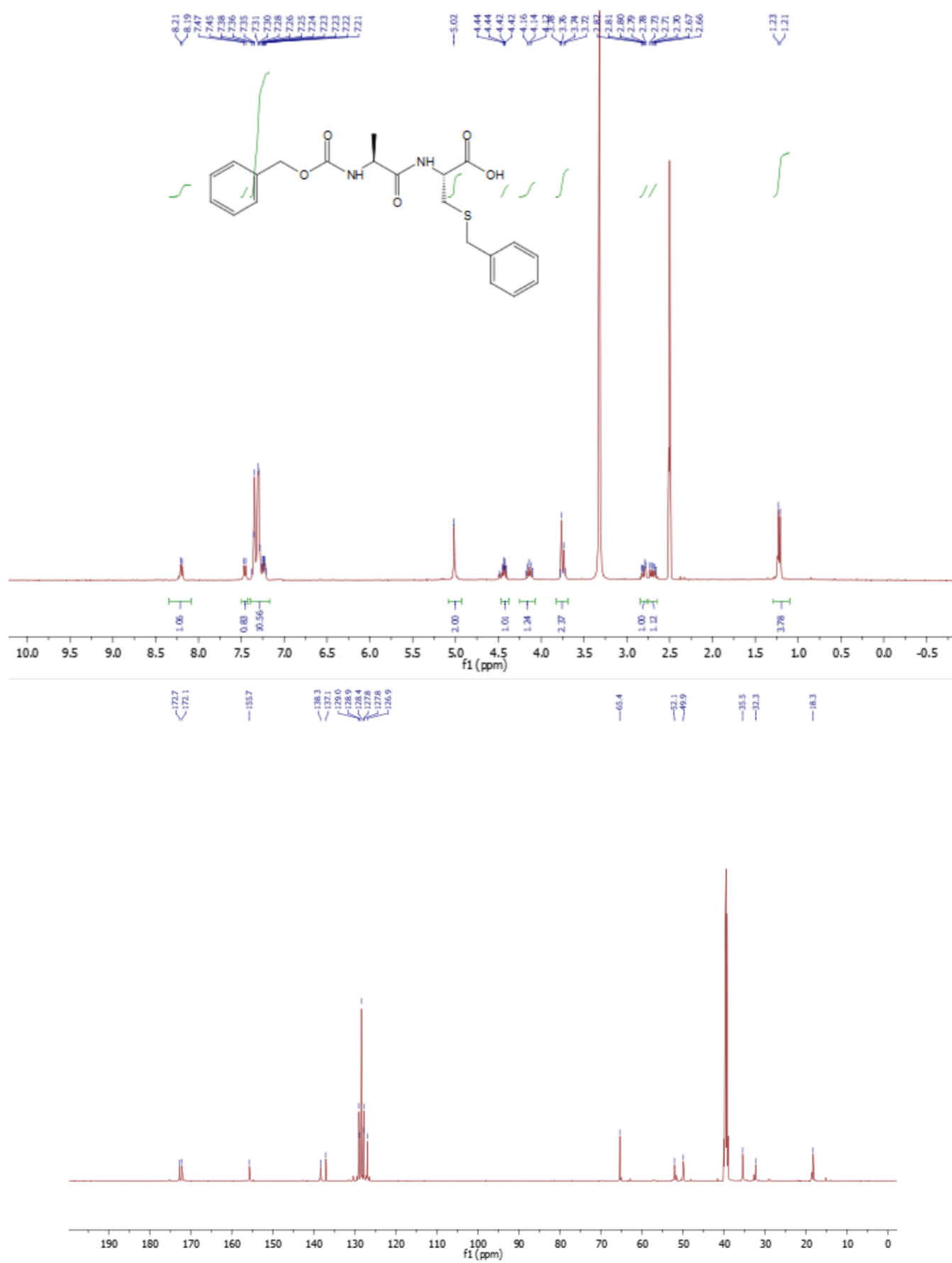
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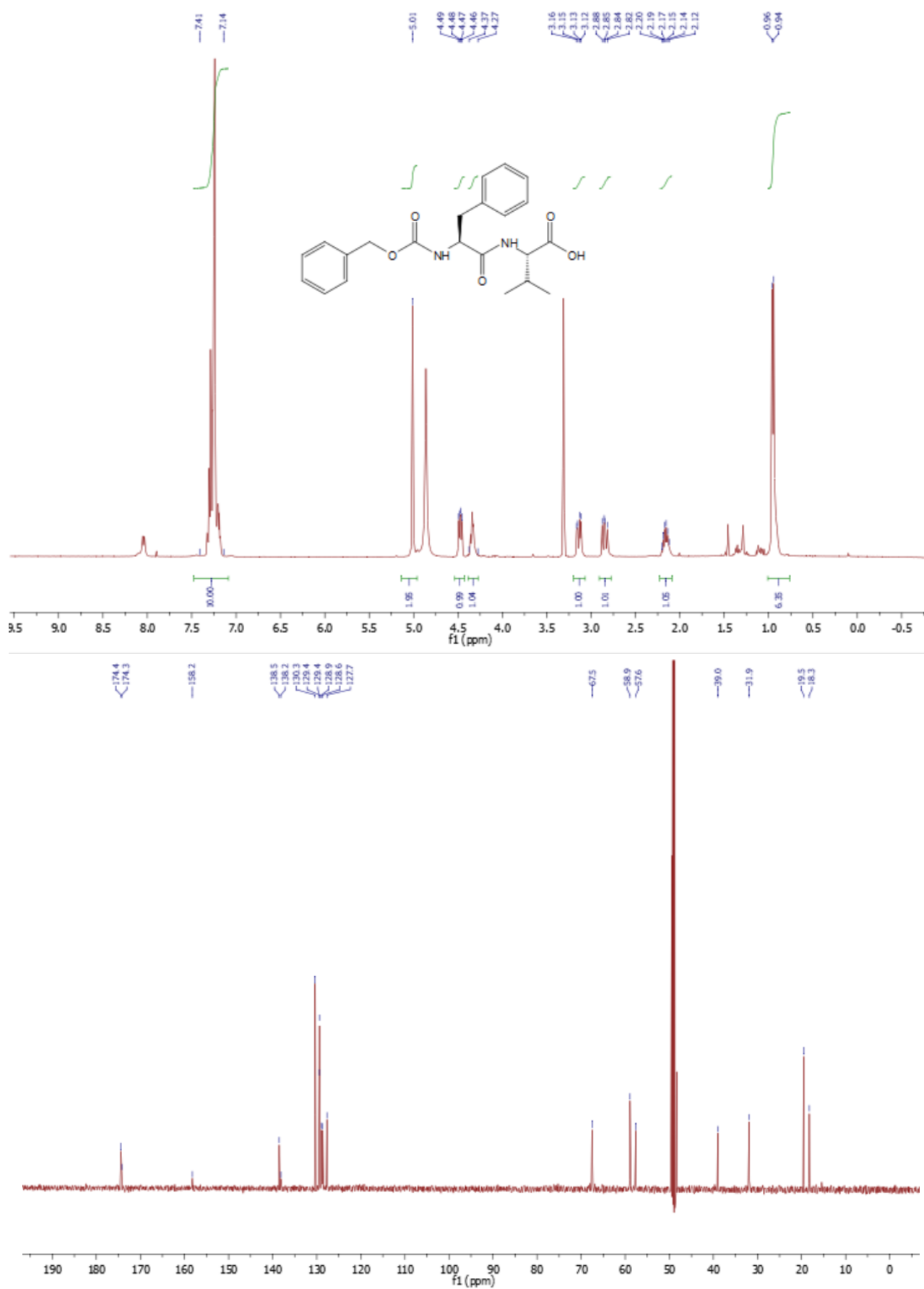
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- Z-Ala-Cys(Bn)-OH



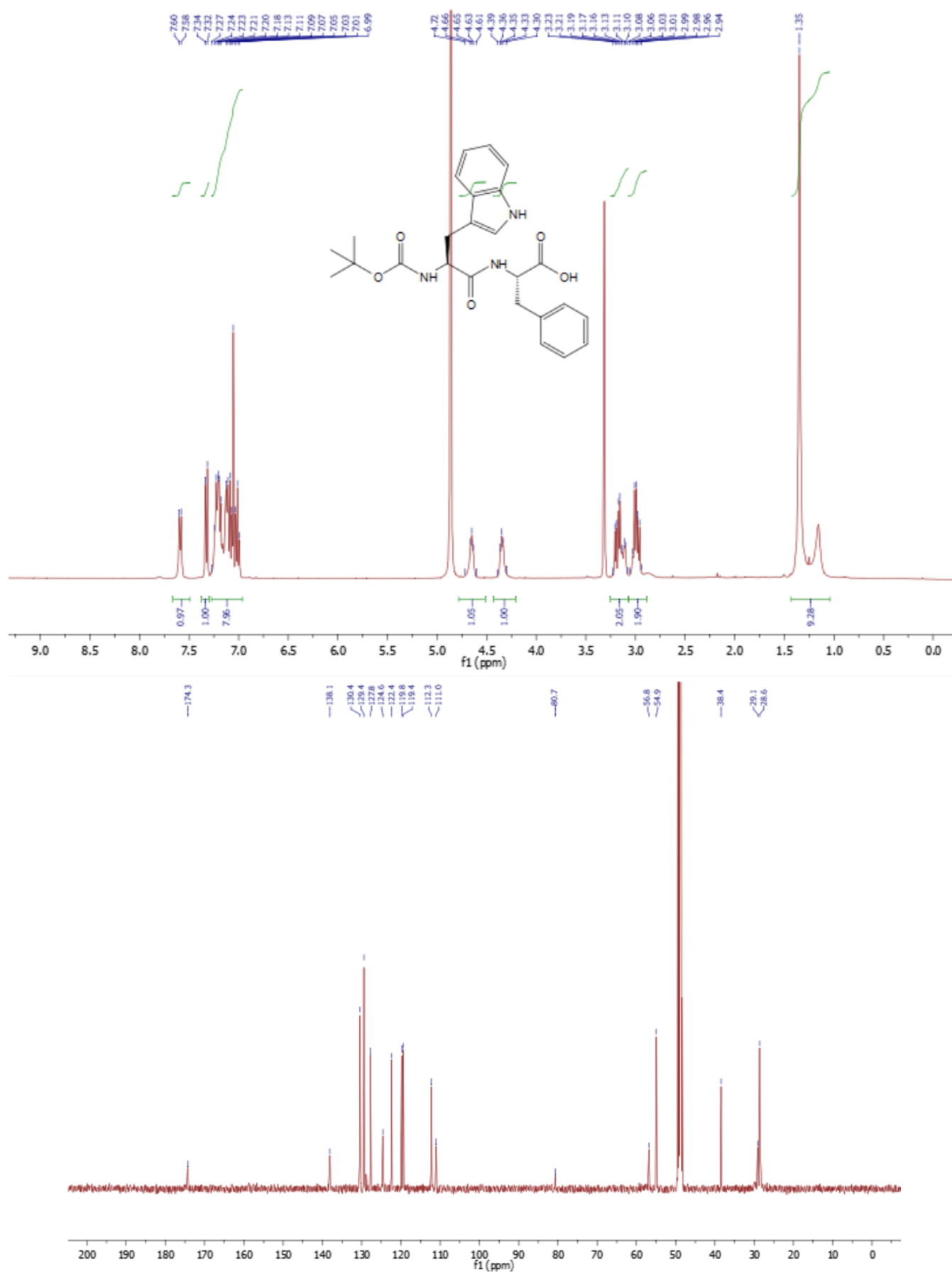
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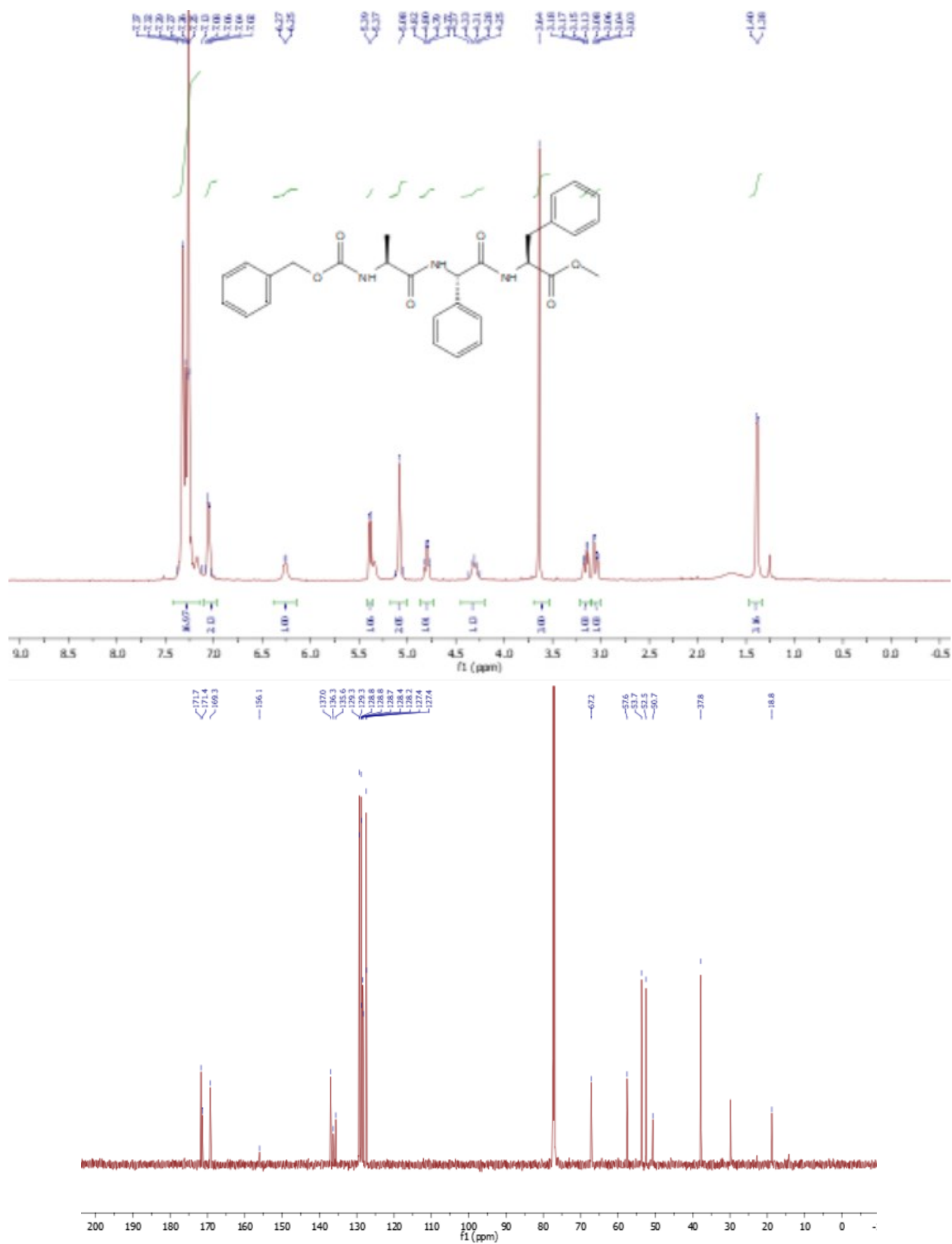
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- Boc-Trp-Phe-OH

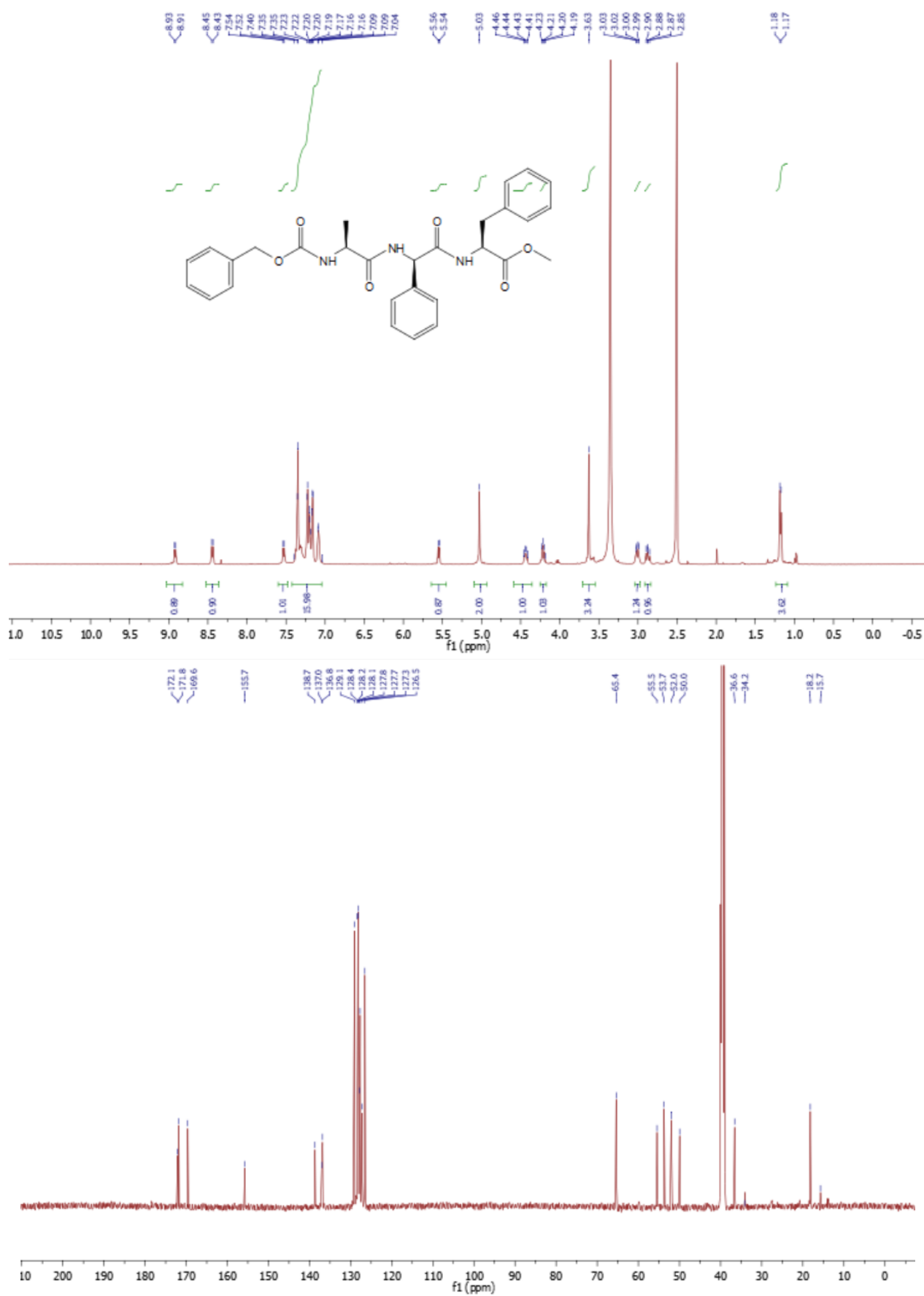


- Z-Ala-Phg-Phe-OMe

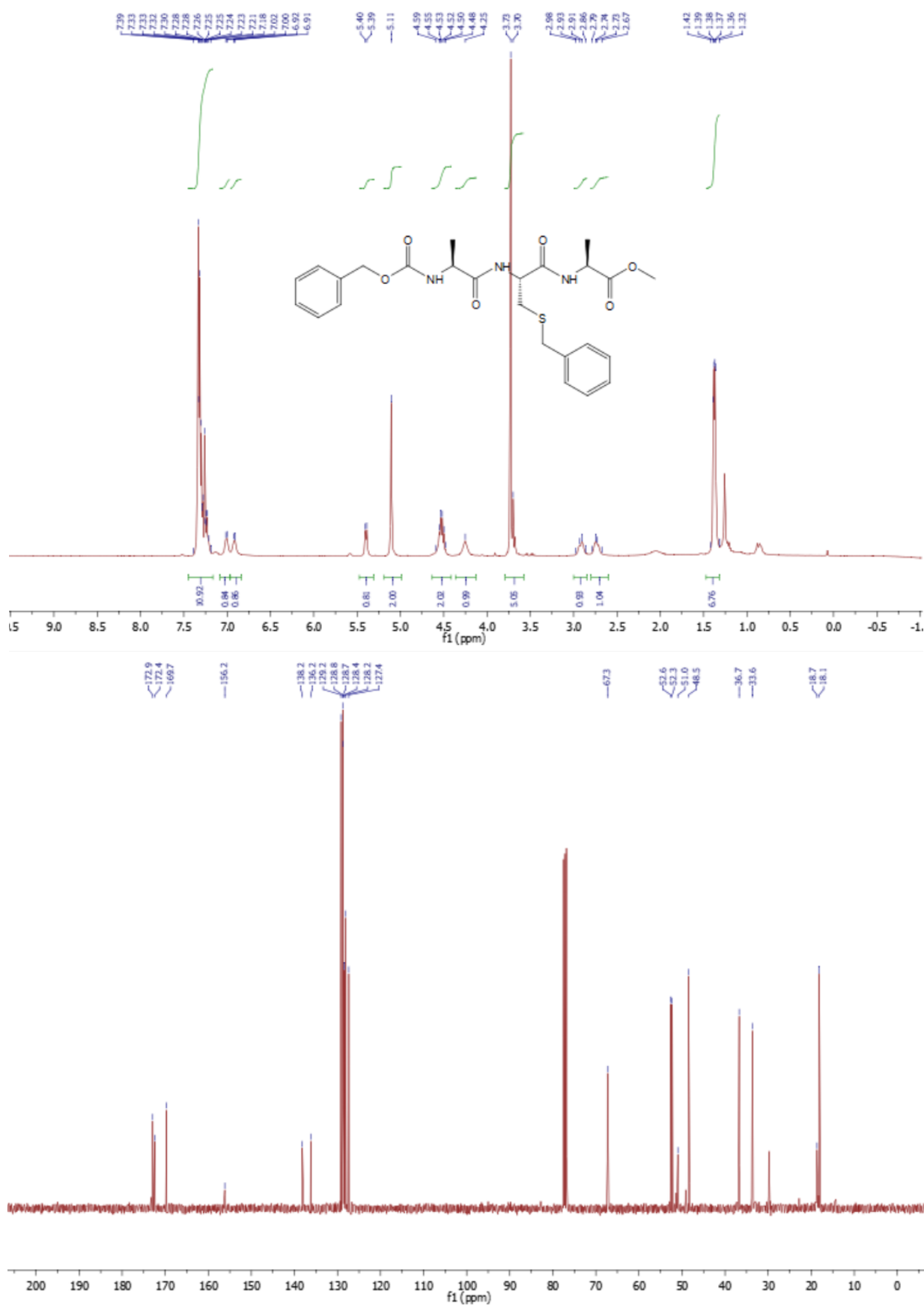




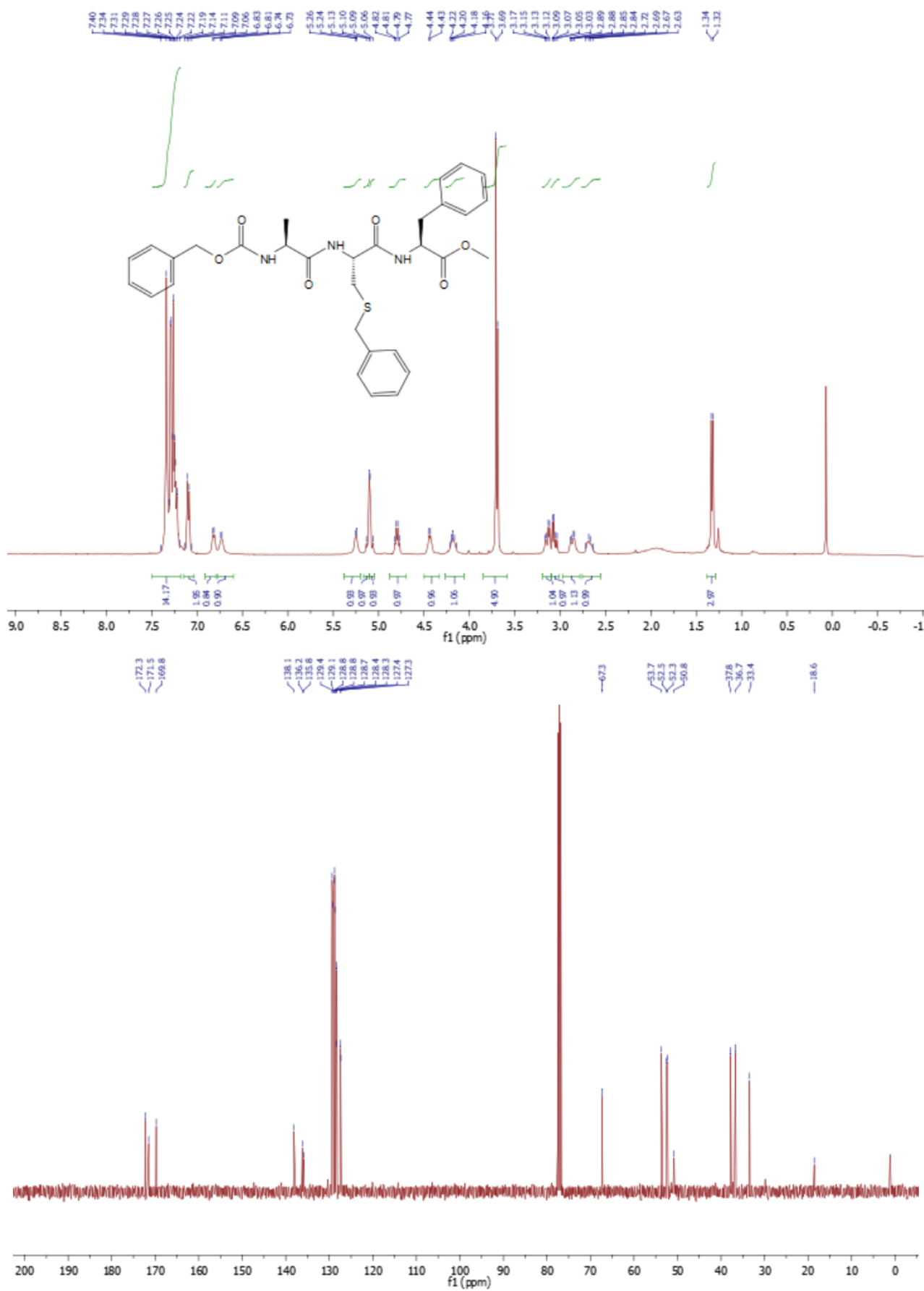
- Z-Ala-D-Phg-Phe-OMe



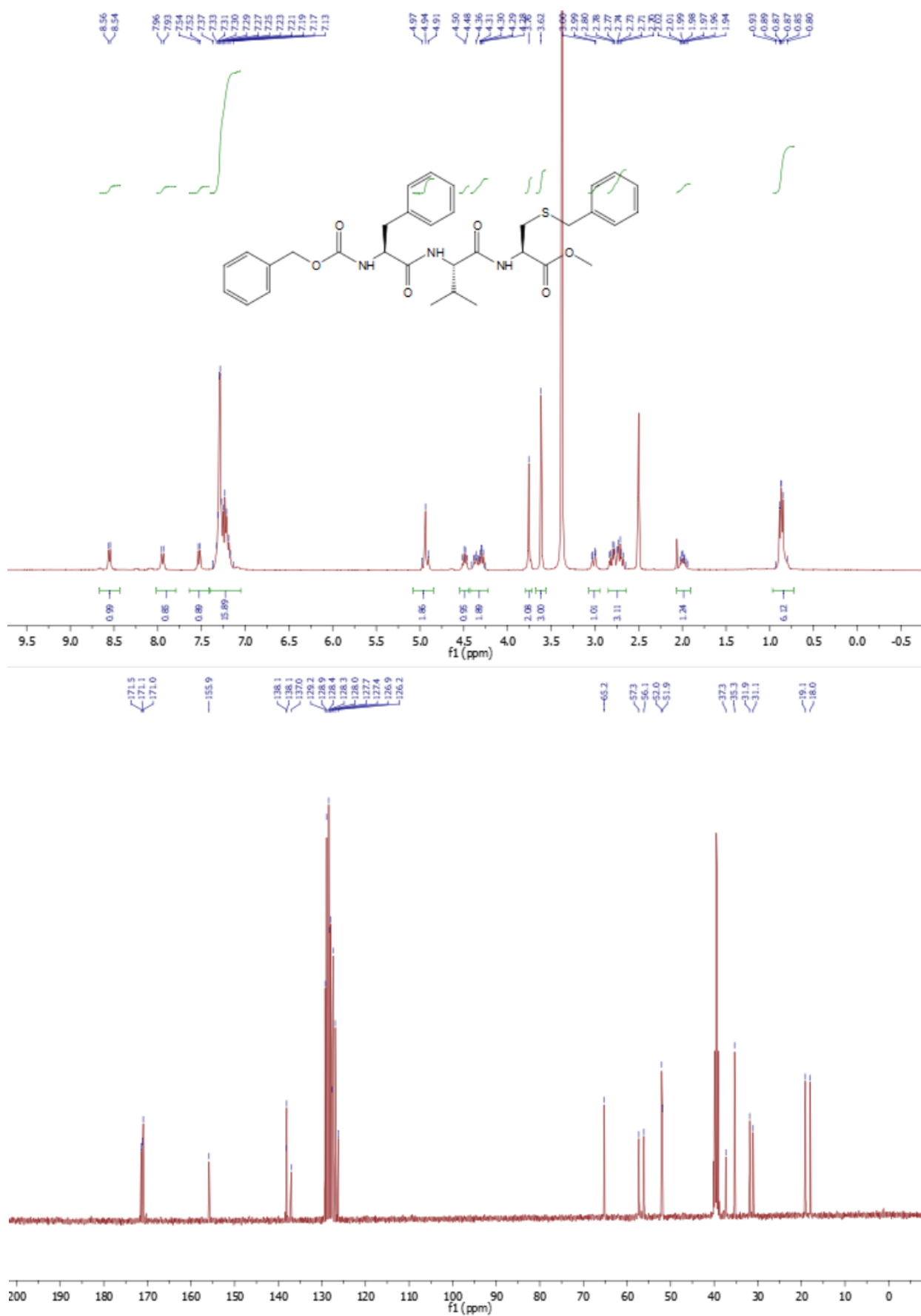
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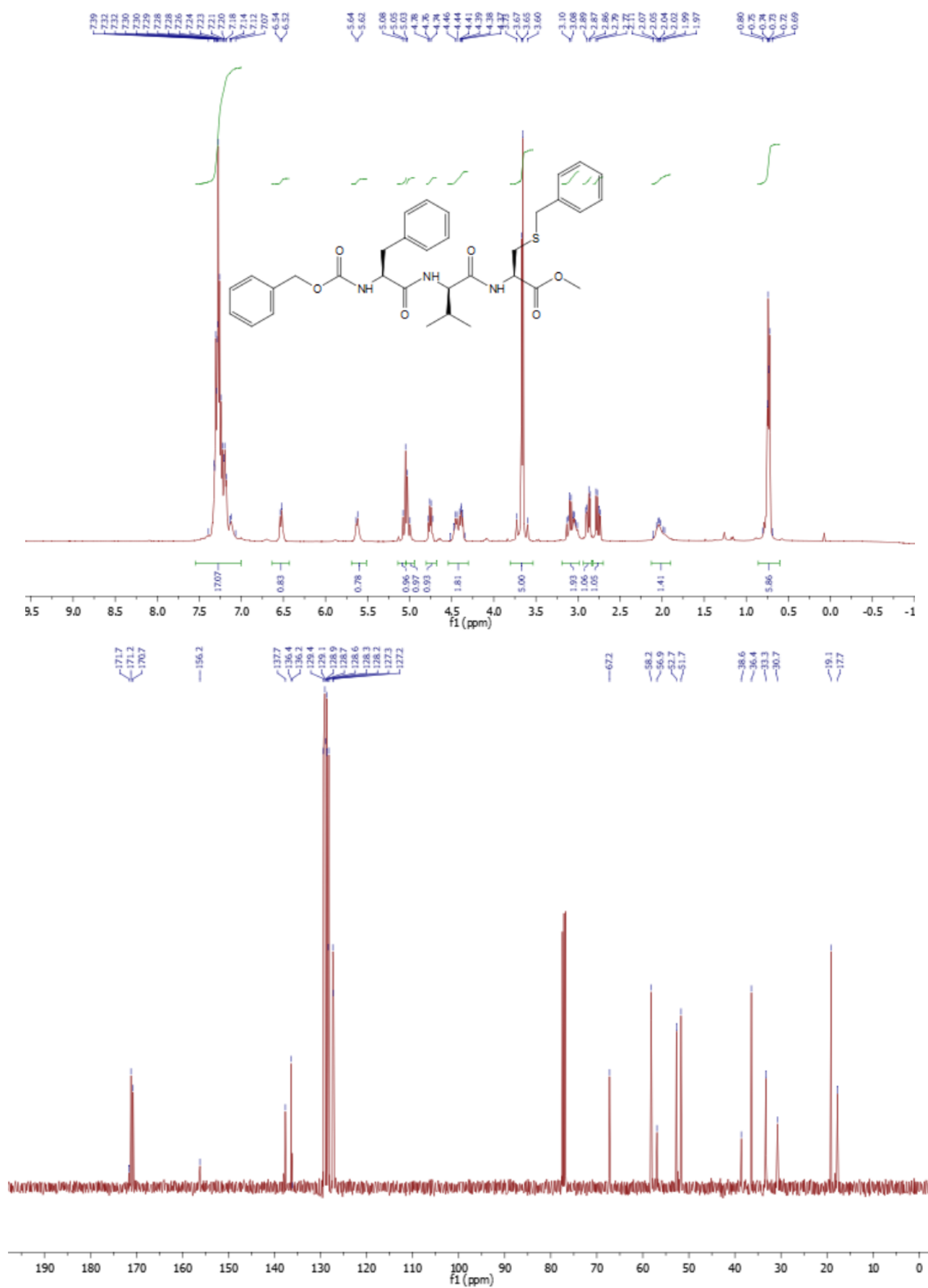
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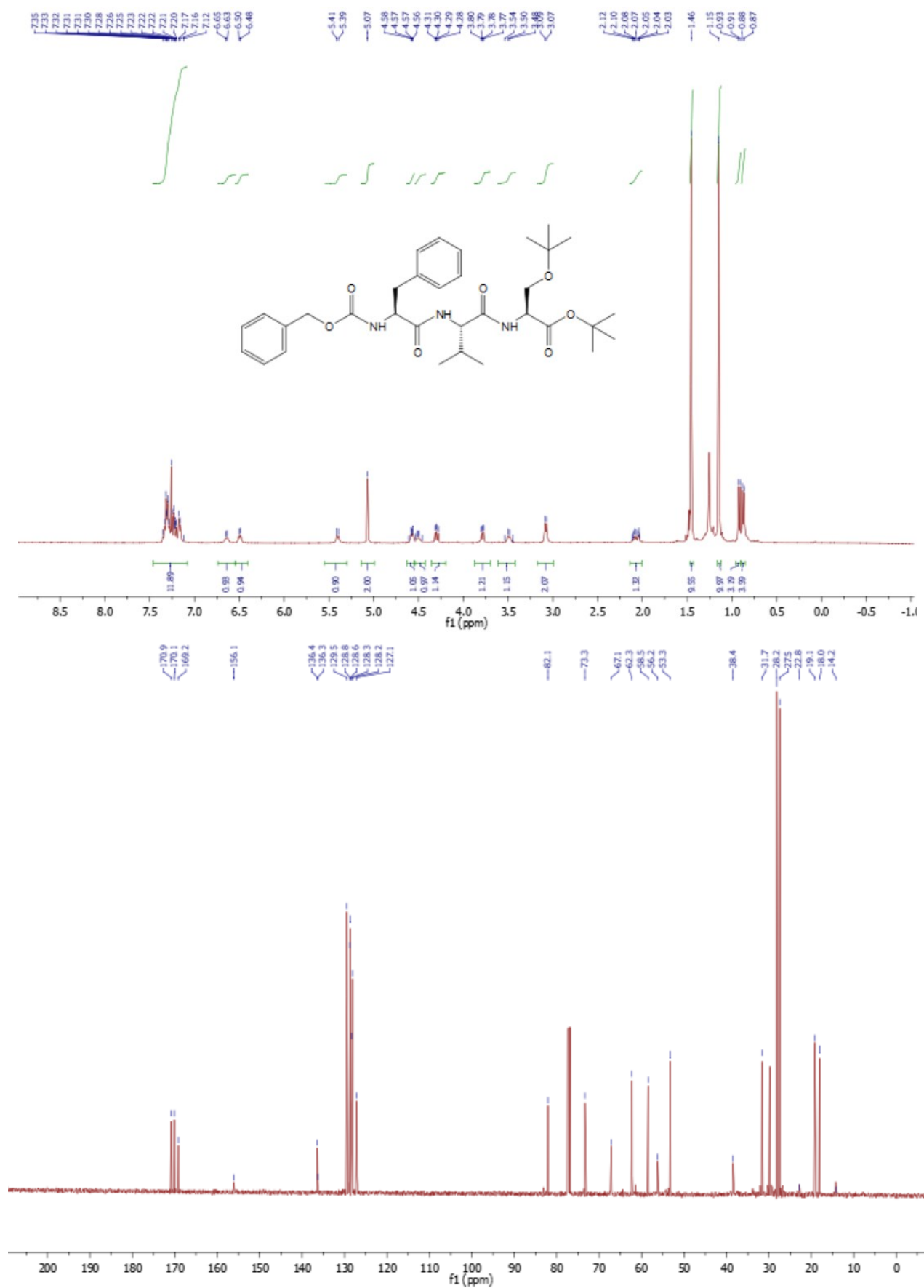
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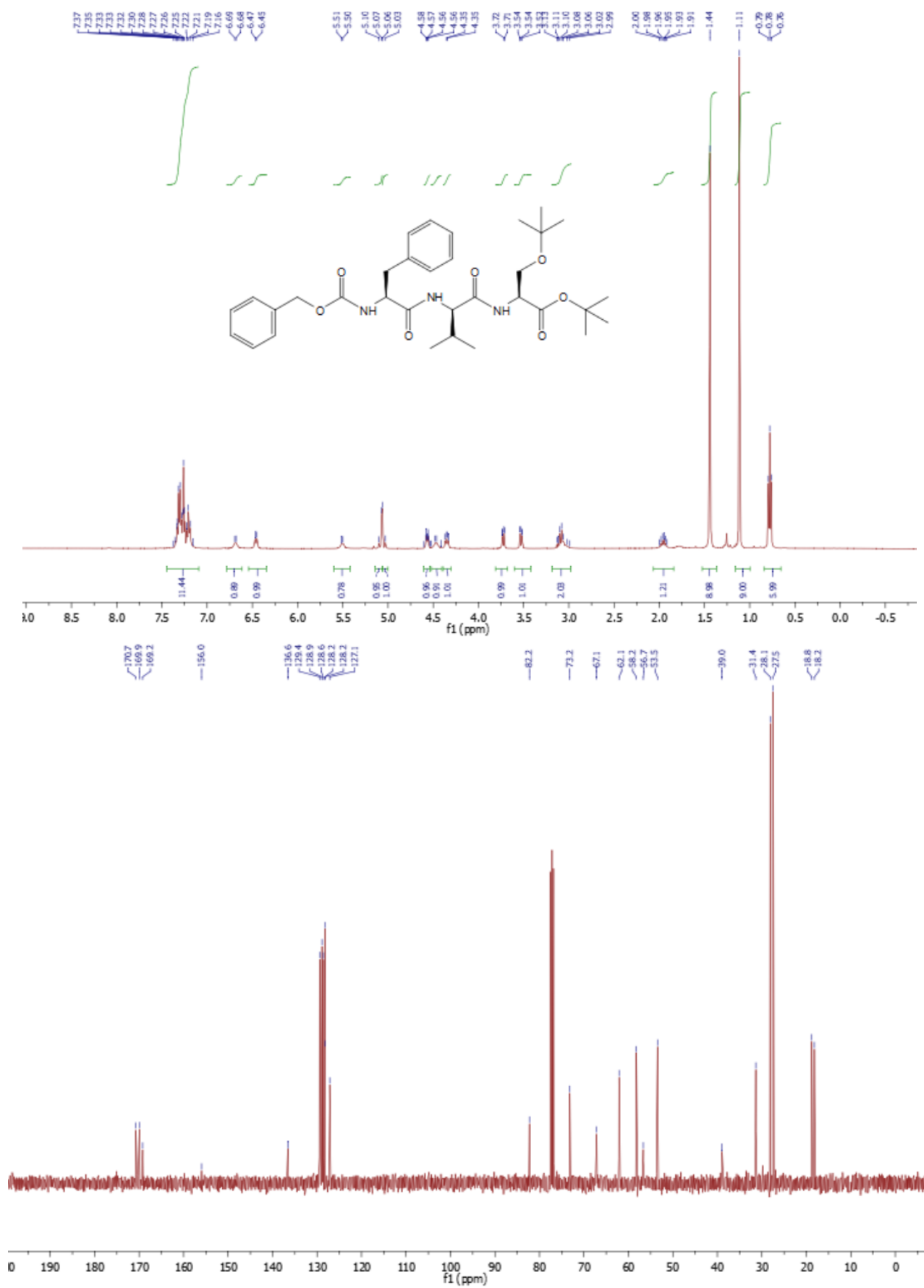
- Z-Phe-D-Val-Cys(Bn)-OMe



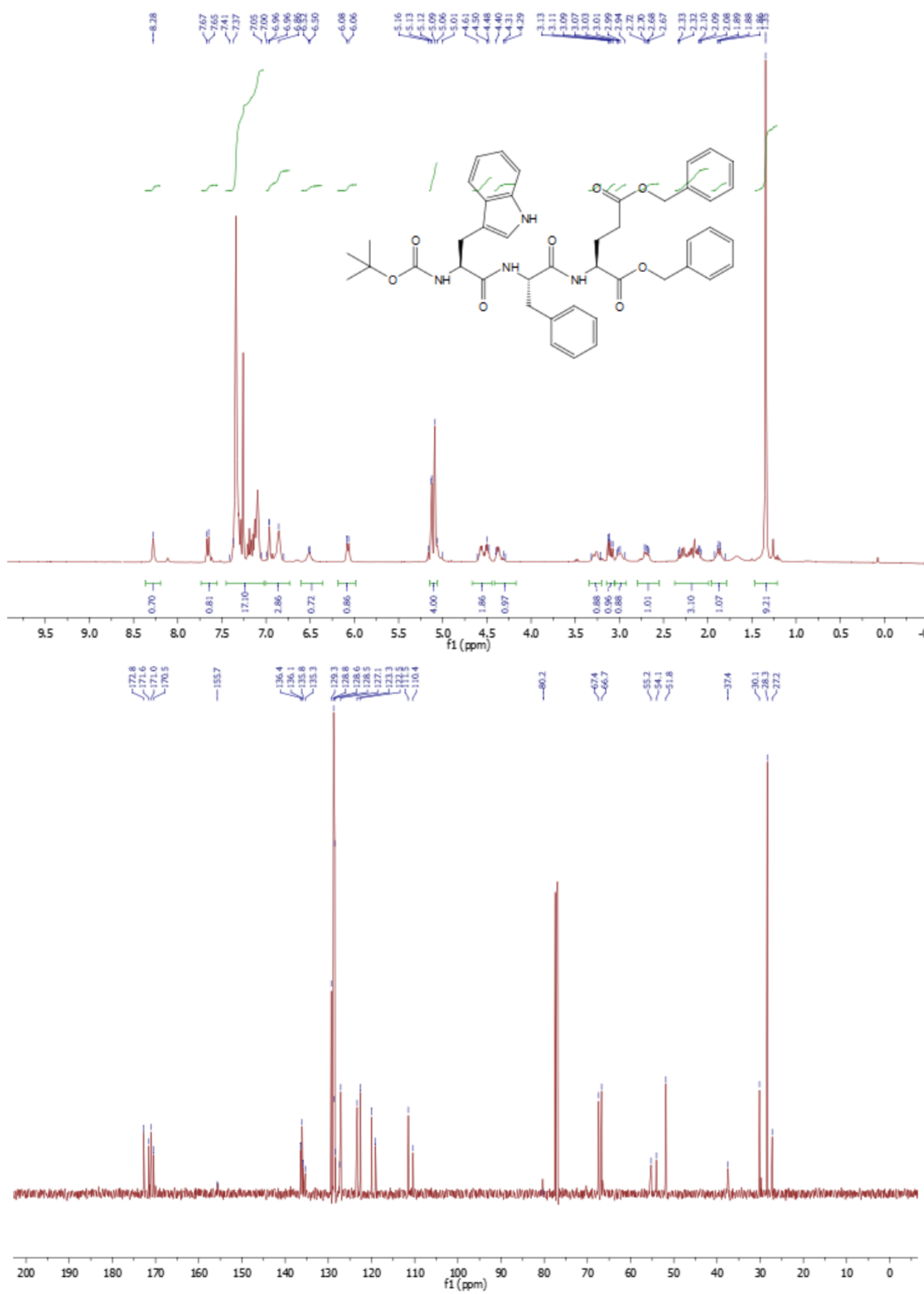
- Z-Phe-Val-Ser(OtBu)-OtBu



- Z-Phe-D-Val-Ser(OTf)-OTfBu

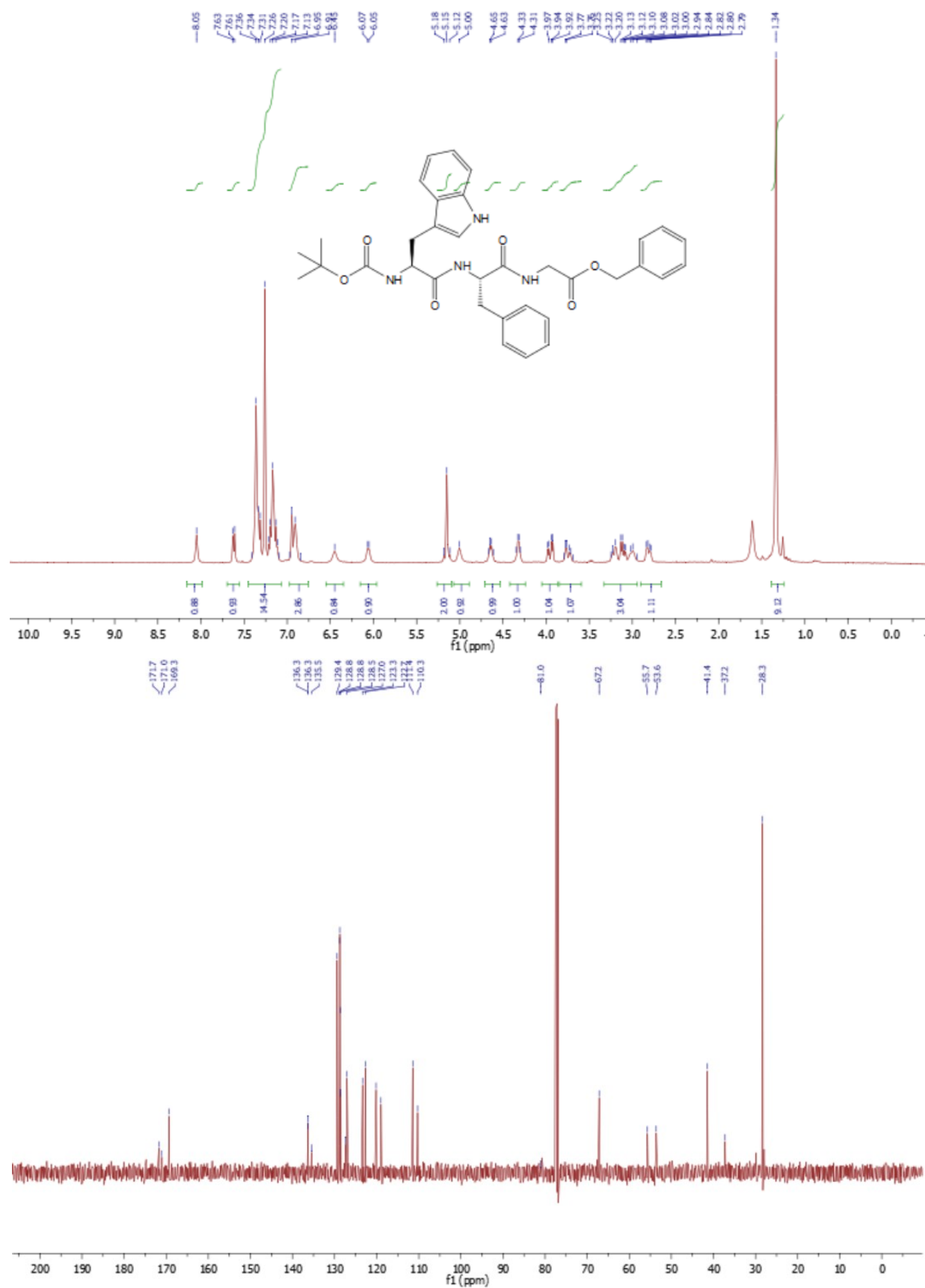


- Z-Trp-Phe-Glu(Bn)-OBn

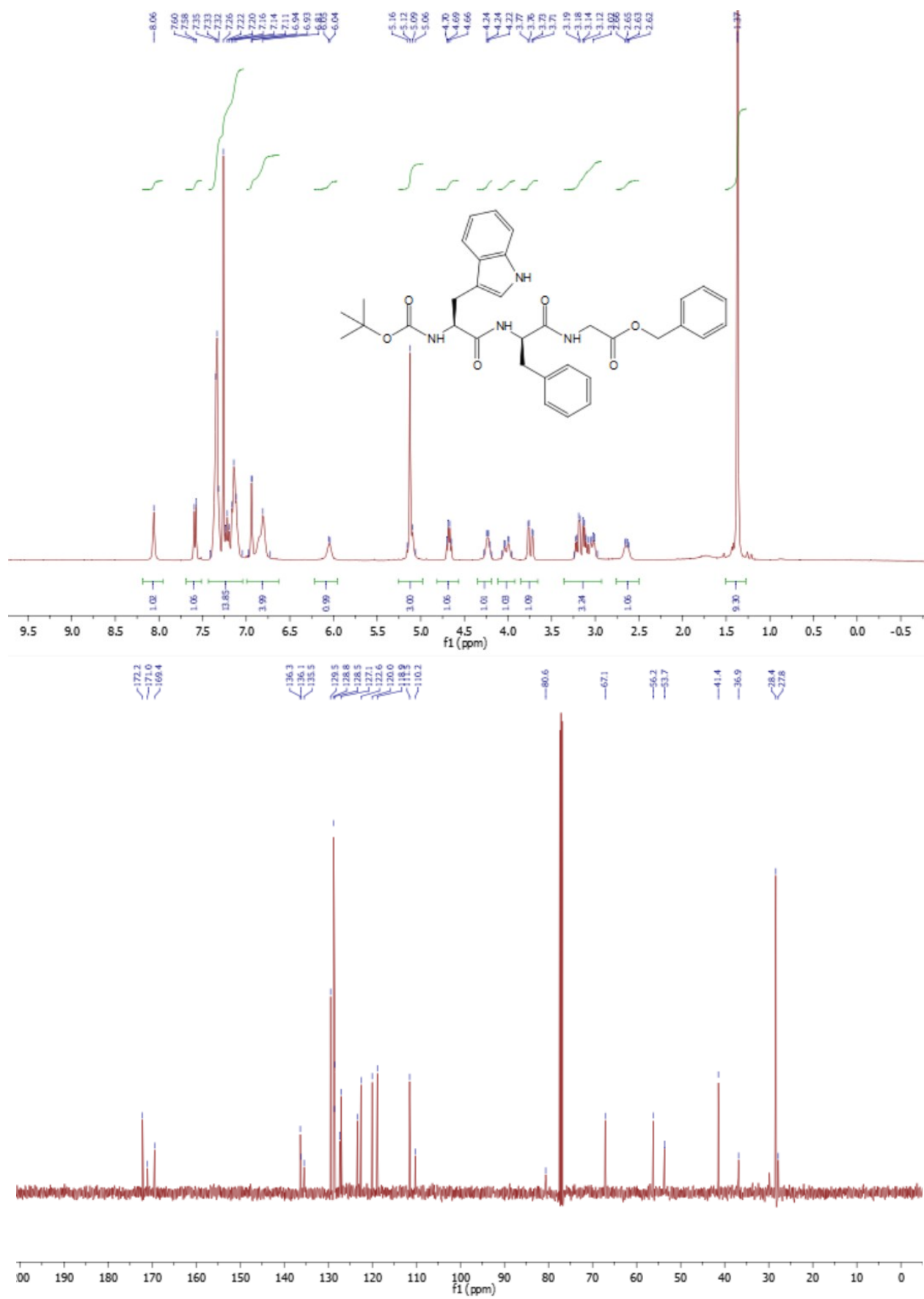




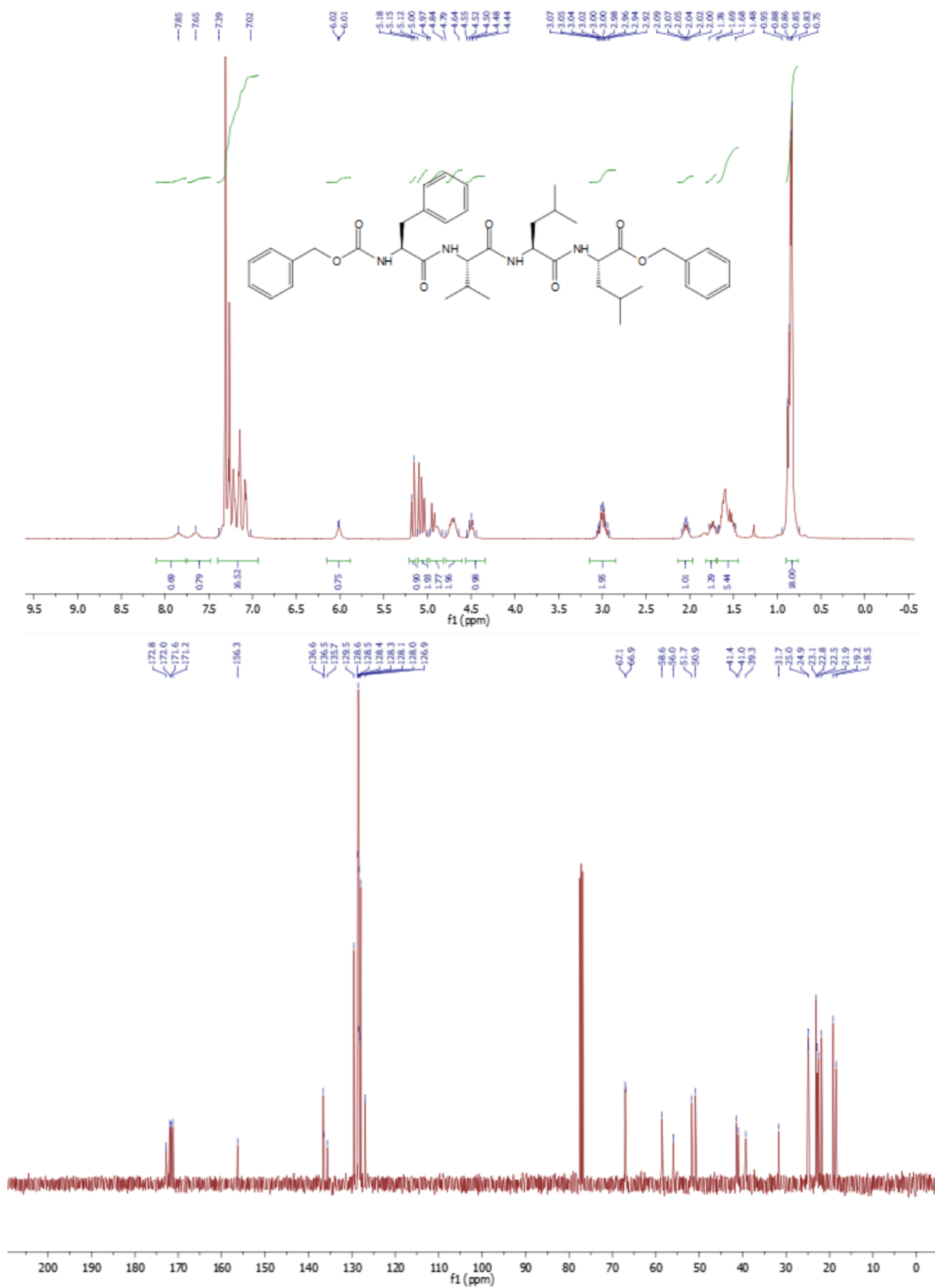
- Boc-Trp-Phe-Gly-OBn



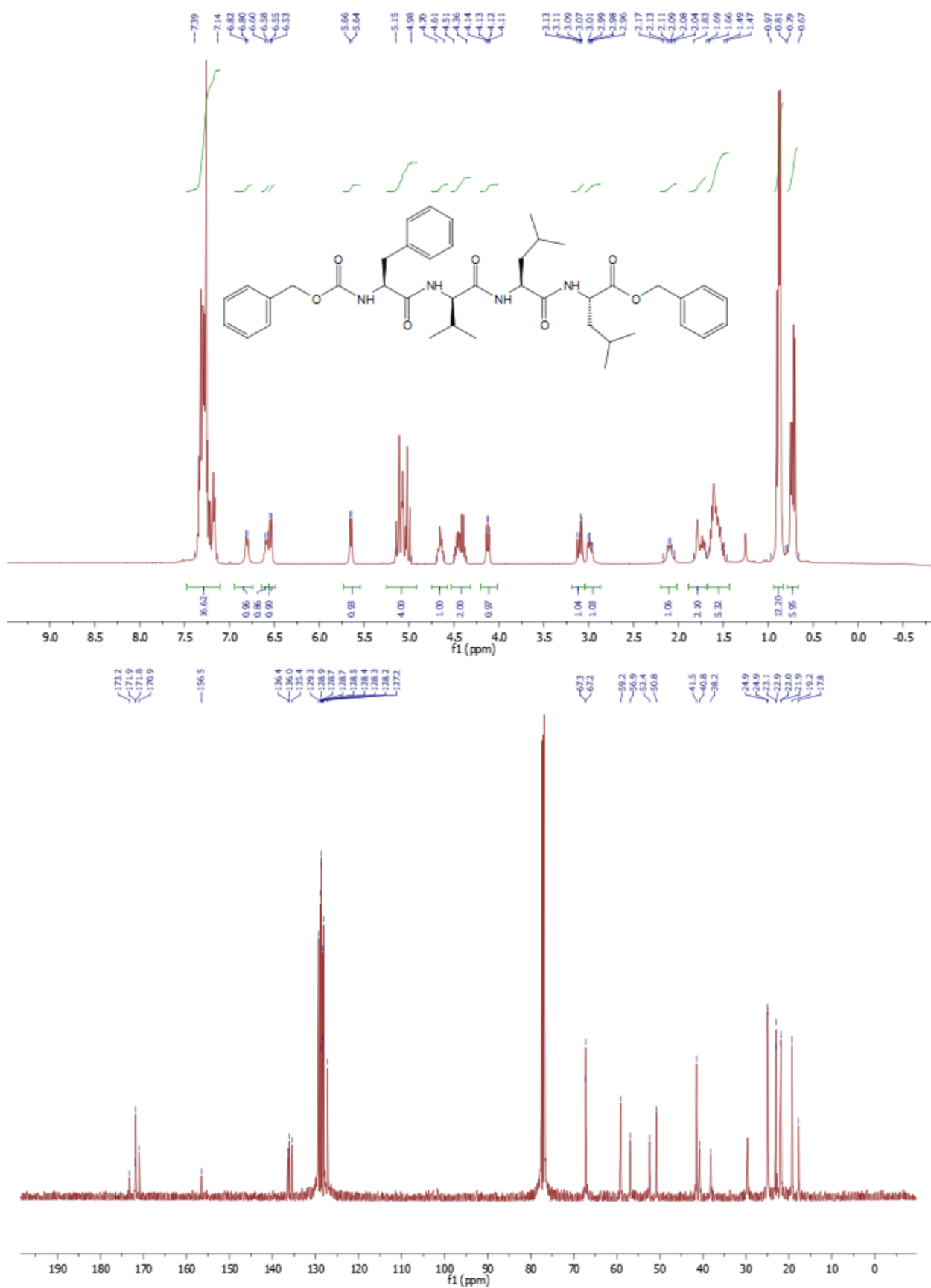
- Boc-Trp-D-Phe-Gly-OBn



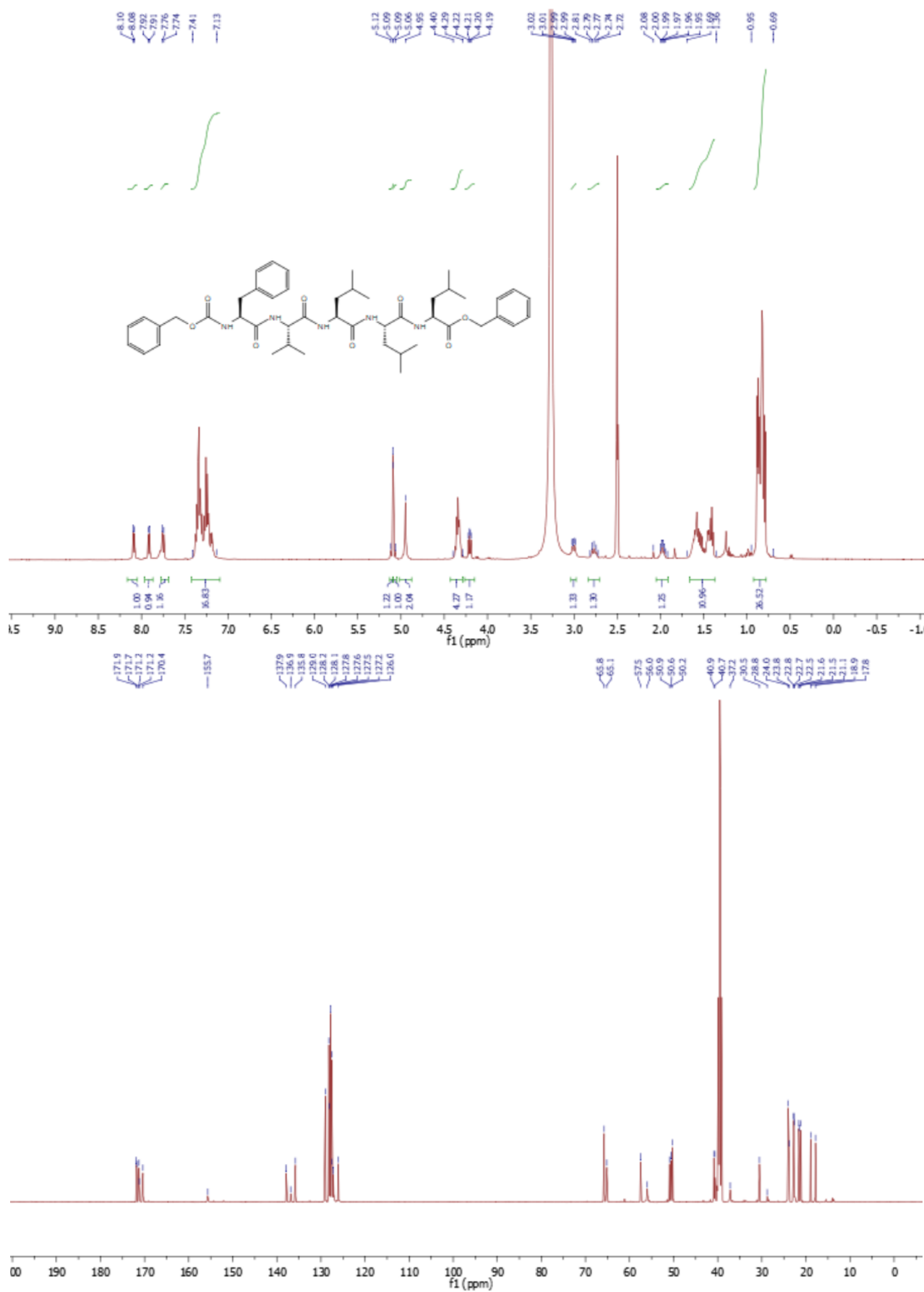
- Z-Phe-Val-Leu<sub>2</sub>-OBn



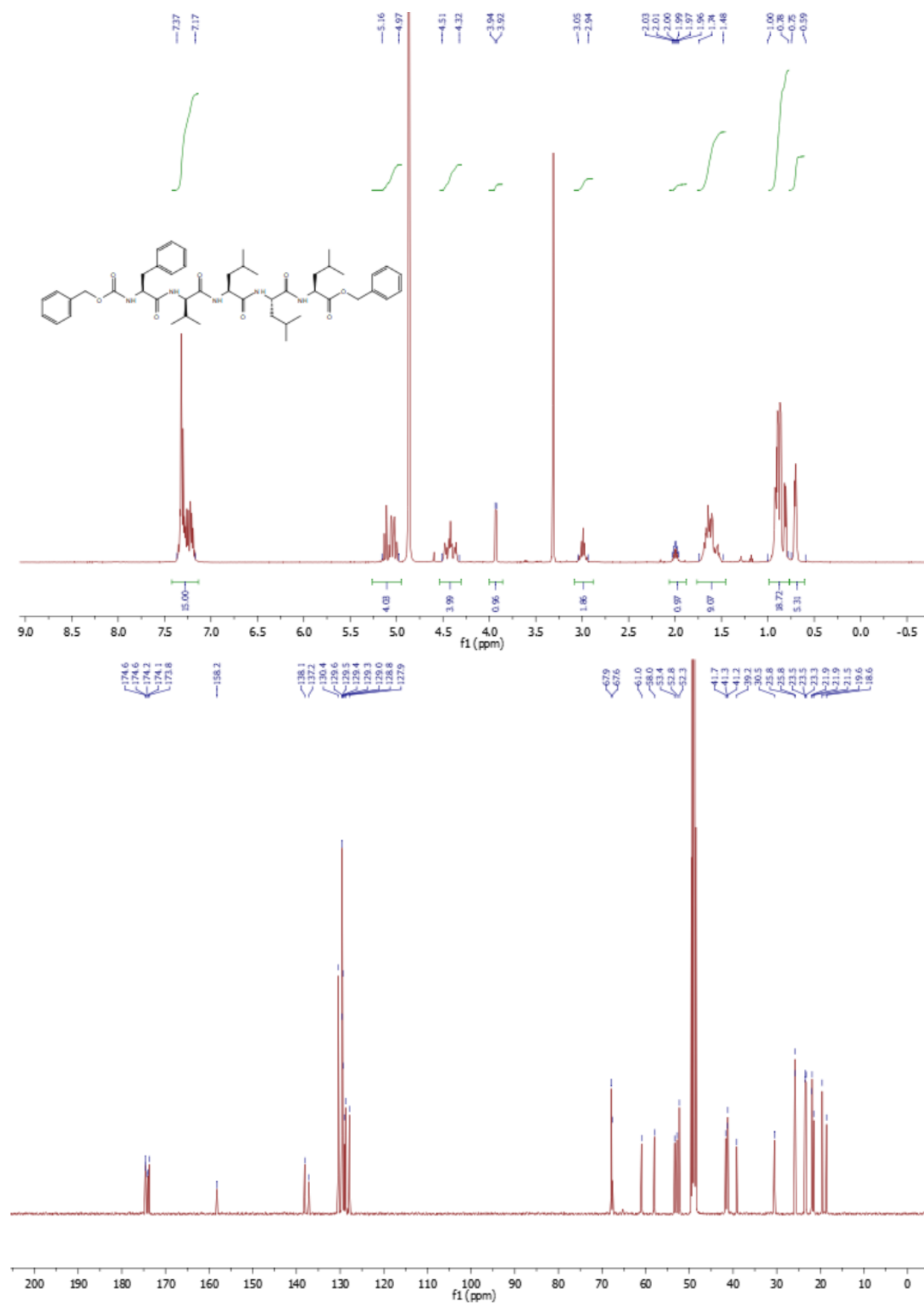
- Z-Phe-D-Val-Leu<sub>2</sub>-OBn



- Z-Phe-Val-Leu<sub>3</sub>-OBn



- Z-Phe-D-Val-Leu<sub>3</sub>-OBn



## HPLC chromatograms

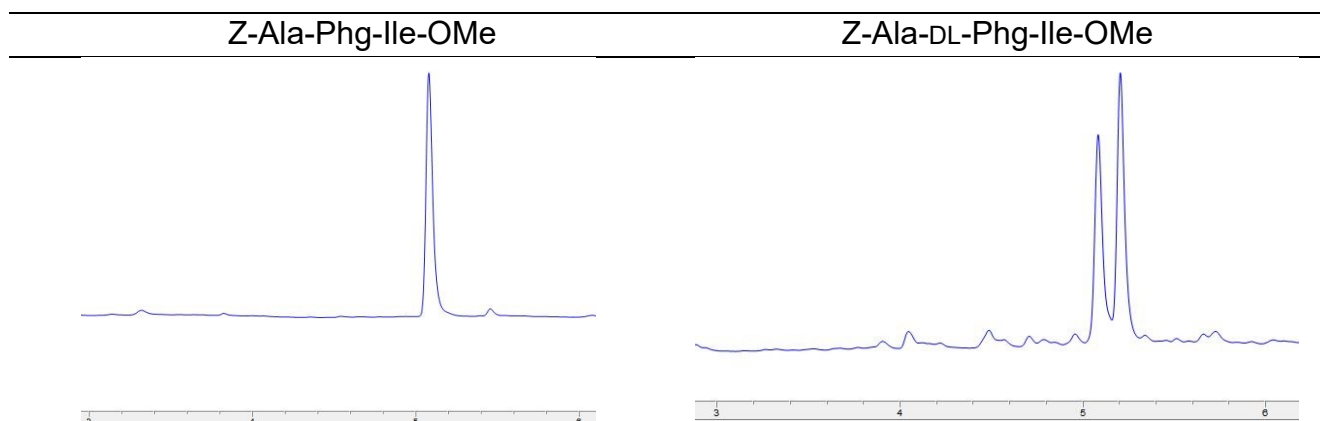


Figure 1: Chromatograms of Z-Ala-Phg-Ile-OMe and Z-Ala-DL-Phg-Ile-OMe

Overlay of Z-Ala-Phg-Ile-OMe (in pink) and  
Z-Ala-DL-Phg-Ile-OMe (in blue)

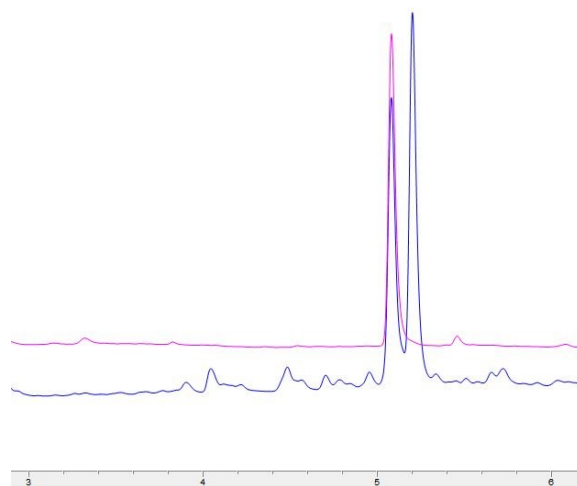
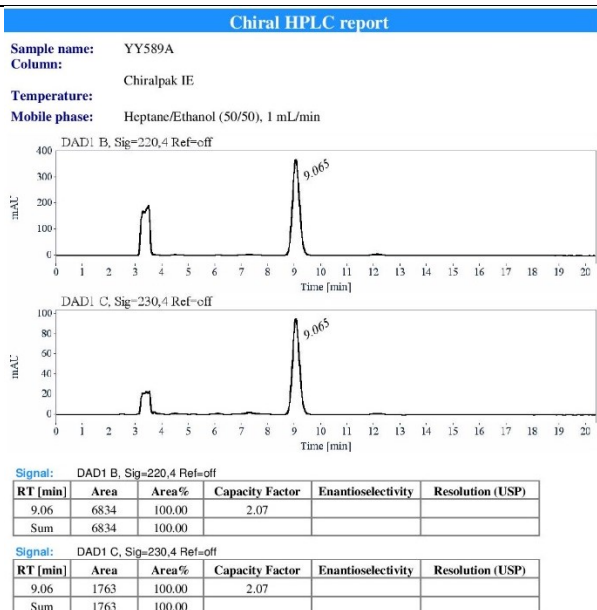


Figure 2: Overlay of Z-Ala-Phg-Ile-OMe and Z-Ala-DL-Phg-Ile-OMe

## Z-Ala-Phg-Phe-OMe



## Z-Ala-D-Phg-Phe-OMe

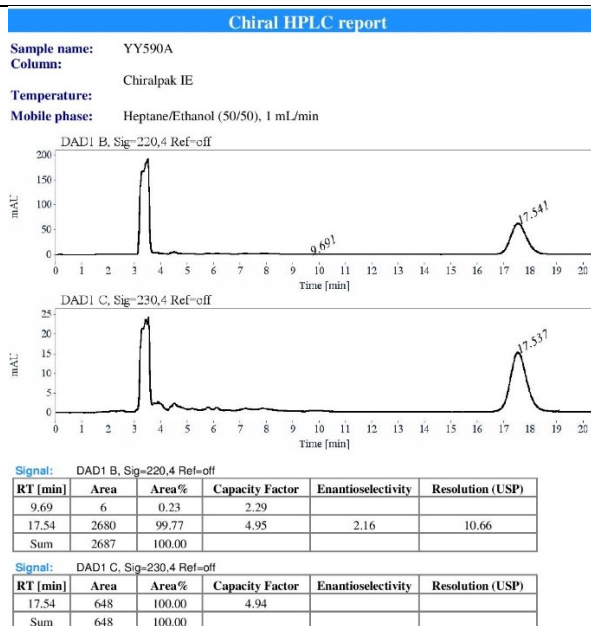
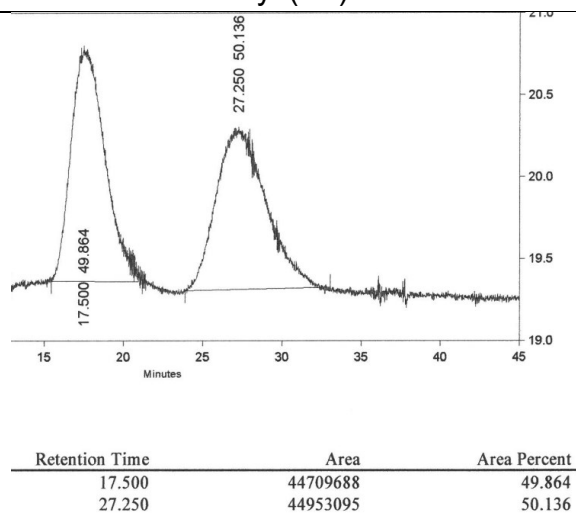


Figure 3: Chromatograms of Z-Ala-Phg-Phe-OMe and Z-Ala-D-Phg-Phe-OMe

## Z-Ala-DL-Cys(Bn)-Ala-OMe



## Z-Ala-Cys(Bn)-Ala-OMe

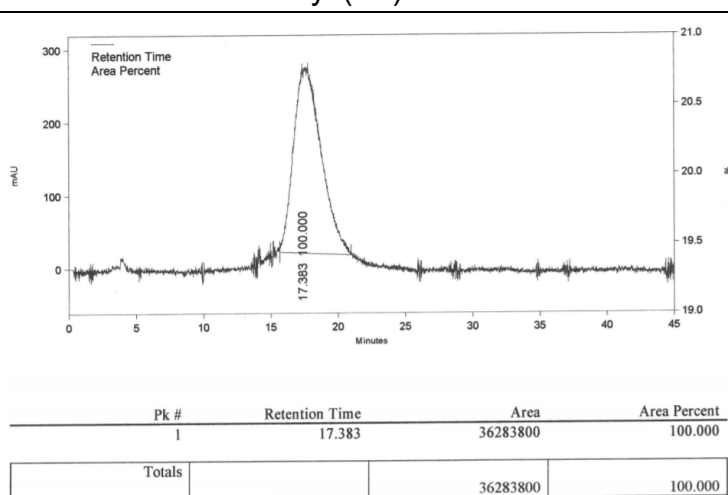
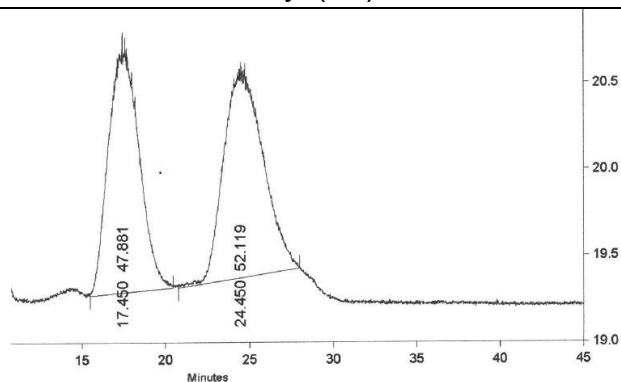


Figure 4: Chromatograms of Z-Ala-DL-Cys(Bn)-Ala-OMe and Z-Ala-Cys(Bn)-Ala-OMe

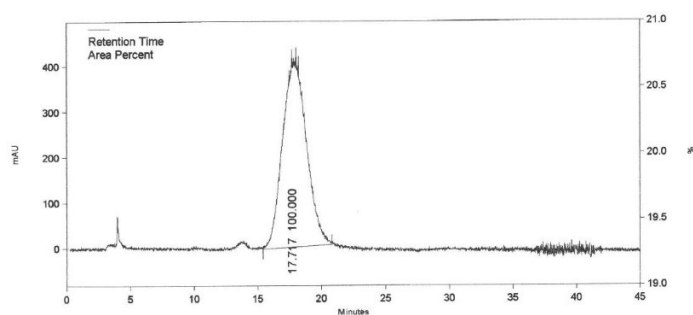


### Z-Ala-DL-Cys(Bn)-Phe-OMe



Retention Time	Area	Area Percent
17.450	76088010	47.881
24.450	82823253	52.119

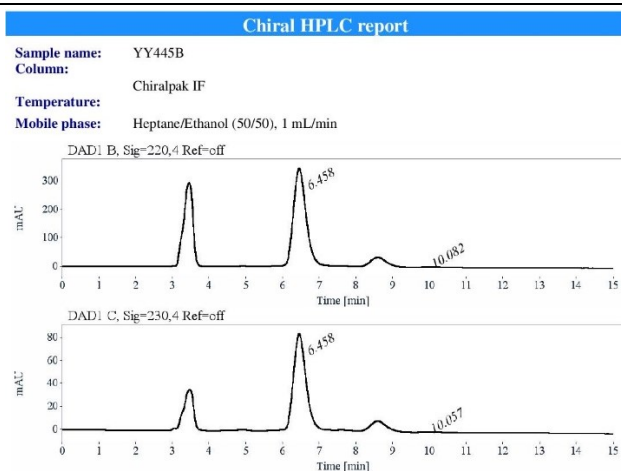
### Z-Ala-Cys(Bn)-Phe-OMe



PK #	Retention Time	Area	Area Percent
1	17.717	54033704	100.000
Totals		54033704	100.000

Figure 5: Chromatograms of Z-Ala-DL-Cys(Bn)-Phe-OMe and Z-Ala-Cys(Bn)-Phe-OMe

### Z-Phe-Val-Cys(Bn)-OMe



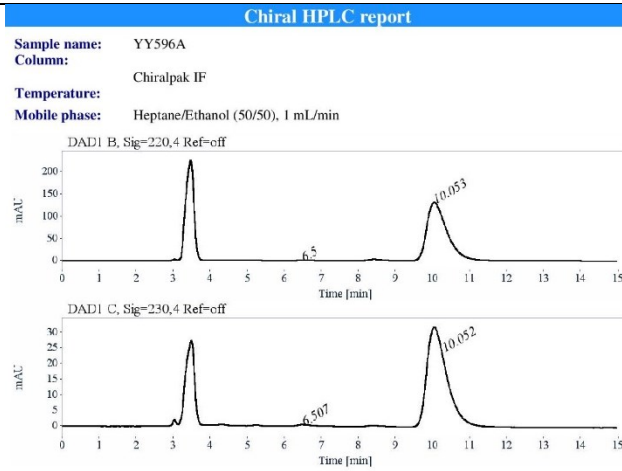
Signal: DAD1 B, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.46	8145	99.24	1.19		
10.08	62	0.76	2.42	2.03	5.02
Sum	8208	100.00			

Signal: DAD1 C, Sig=230,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.46	1972	99.48	1.19		
10.06	10	0.52	2.41	2.03	5.21
Sum	1982	100.00			

### Z-Phe-D-Val-Cys(Bn)-OMe



Signal: DAD1 B, Sig=220,4 Ref=off

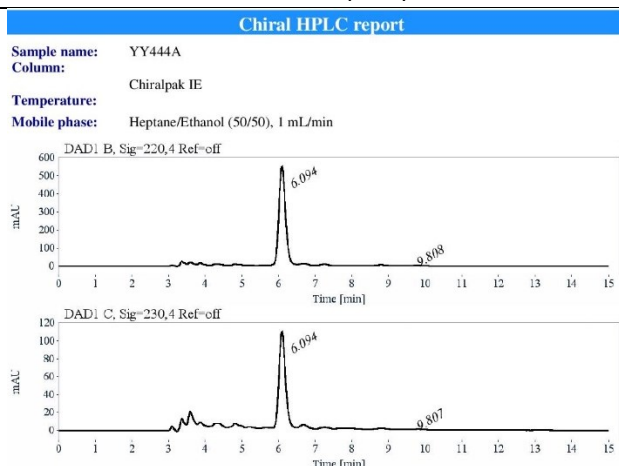
RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.50	25	0.51	1.20		
10.05	4940	99.49	2.41	2.00	4.90
Sum	4965	100.00			

Signal: DAD1 C, Sig=230,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.51	10	0.85	1.21		
10.05	1205	99.15	2.41	2.00	4.95
Sum	1216	100.00			

Figure 6: Chromatograms of Z-Phe-Val-Cys(Bn)-OMe and Z-Phe-D-Val-Cys(Bn)-OMe

## Z-Phe-Val-Ser(tBu)-OtBu



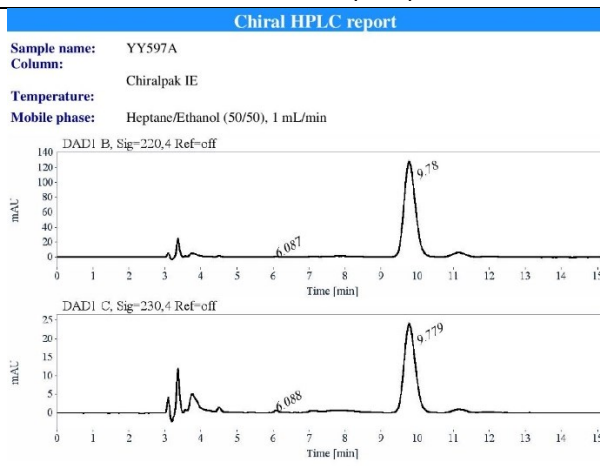
Signal: DAD1 B, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.09	6769	99.23	1.07		
9.81	52	0.77	2.32	2.18	9.41
Sum	6821	100.00			

Signal: DAD1 C, Sig=230,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.09	1311	99.22	1.07		
9.81	10	0.78	2.32	2.18	9.94
Sum	1321	100.00			

## Z-Phe-D-Val-Ser(tBu)-OtBu



Signal: DAD1 B, Sig=220,4 Ref=off

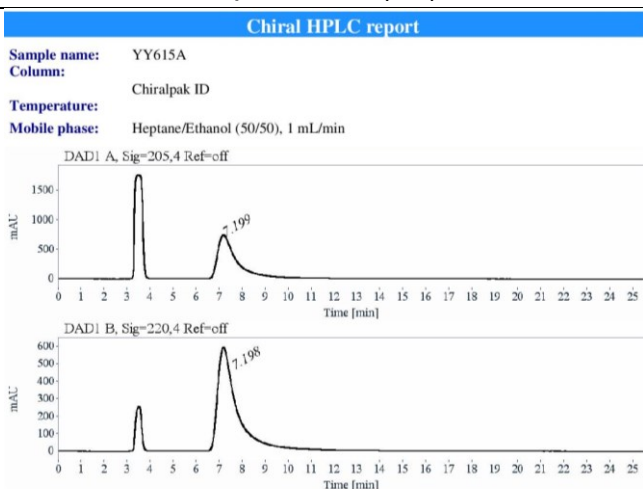
RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.09	13	0.45	1.06		
9.78	2797	99.55	2.32	2.18	9.16
Sum	2810	100.00			

Signal: DAD1 C, Sig=230,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.09	4	0.83	1.06		
9.78	523	99.17	2.31	2.18	9.49
Sum	528	100.00			

Figure 7: Chromatograms of Z-Phe-Val-Ser(OtBu)-OtBu and Z-Phe-D-Val-Ser(OtBu)-OtBu

## Boc-Trp-Phe-Glu(Bn)-OBn



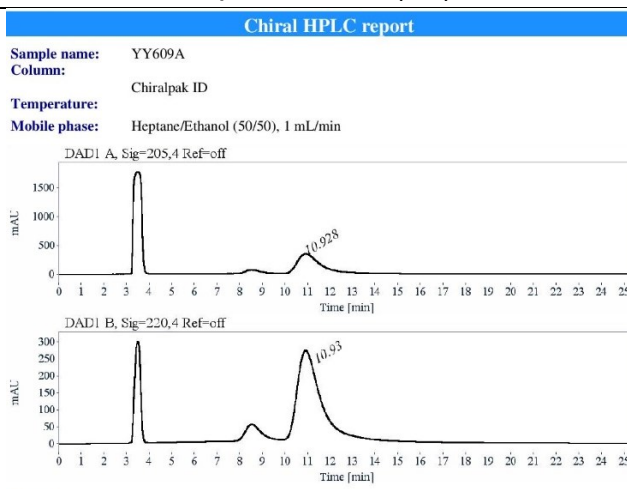
Signal: DAD1 A, Sig=205,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
7.20	40077	100.00	1.44		
Sum	40077	100.00			

Signal: DAD1 B, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
7.20	31797	100.00	1.44		
Sum	31797	100.00			

## Boc-Trp-D-Phe-Glu(Bn)-OBn



Signal: DAD1 A, Sig=205,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
10.93	24481	100.00	2.70		
Sum	24481	100.00			

Signal: DAD1 B, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
10.93	19071	100.00	2.70		
Sum	19071	100.00			

Figure 8: Chromatograms of Boc-Trp-Phe-Glu(Bn)-OBn and Boc-Trp-D-Phe-Glu(Bn)-OBn

## Co-injection of Boc-Trp-Phe-Glu(Bn)-OBn and Boc-Trp-D-Phe-Glu(Bn)-OBn

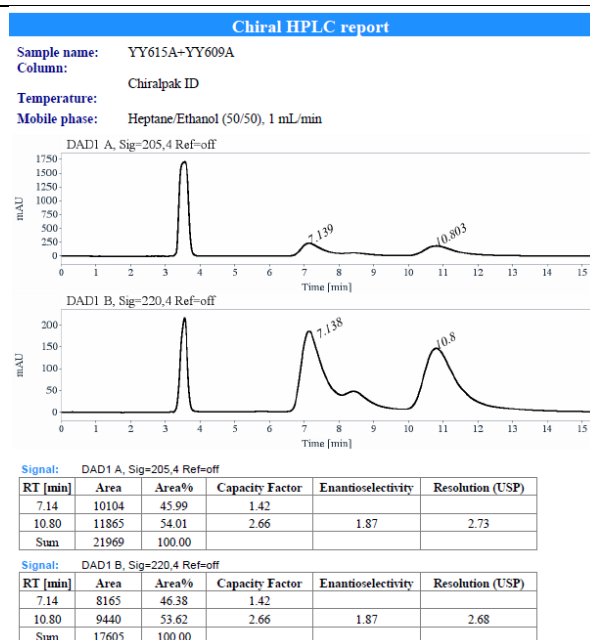


Figure 9: Chromatogram of the co-injection of Boc-Trp-Phe-Glu(Bn)-OBn  
and Boc-Trp-D-Phe-Glu(Bn)-OBn

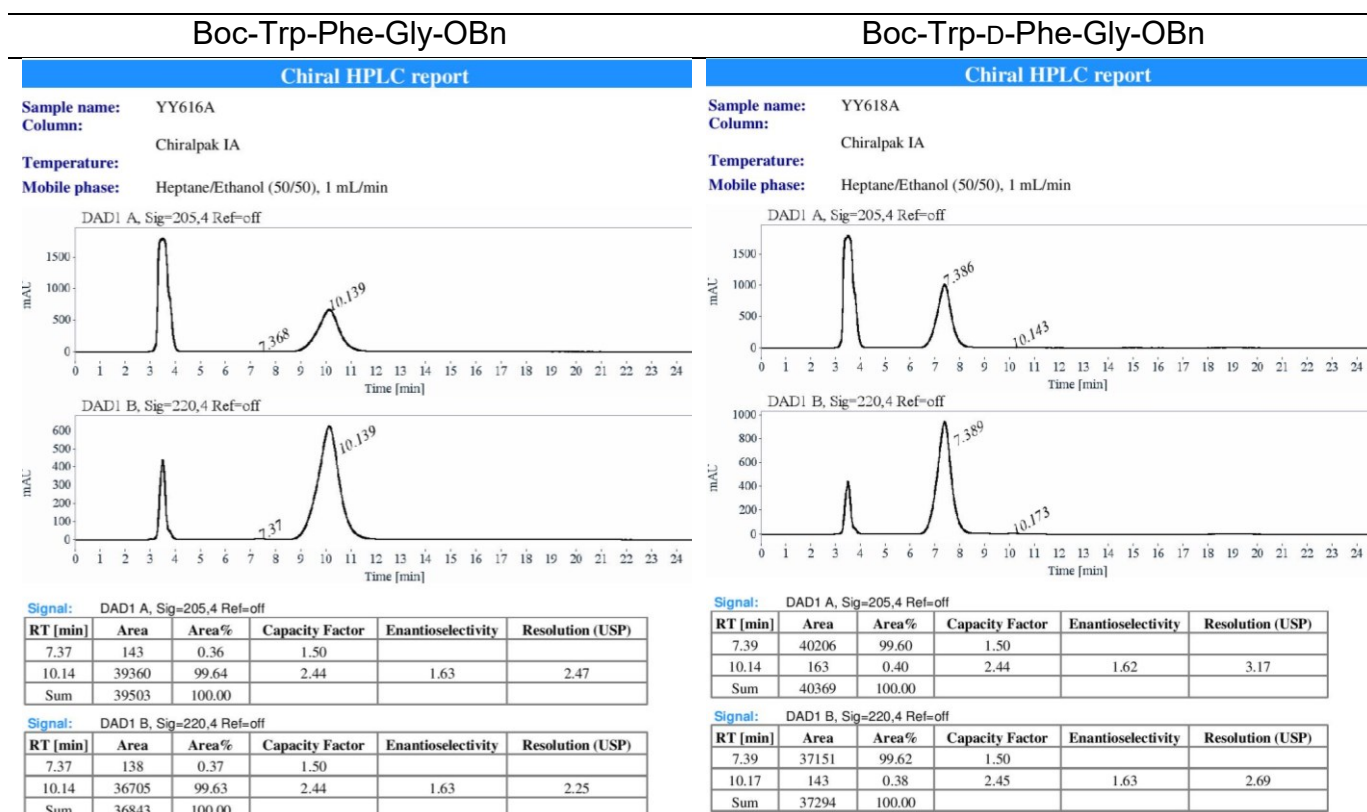
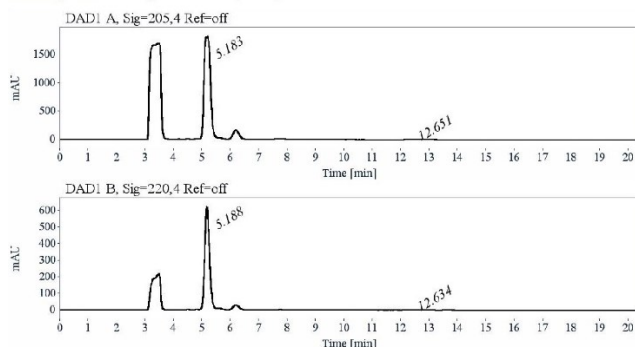


Figure 10: Chromatograms of Boc-Trp-Phe-Gly-OBn and Boc-Trp-D-Phe-Gly-OBn

## Z-Phe-Val-Leu<sub>2</sub>-OBn

### Chiral HPLC report

Sample name: YY599A  
Column: Chiralpak IE  
Temperature:  
Mobile phase: Heptane/Ethanol (50/50), 1 mL/min



Signal: DAD1 A, Sig=205,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
5.18	28357	99.48	0.76		
12.65	149	0.52	3.29	4.34	13.15
Sum	28507	100.00			

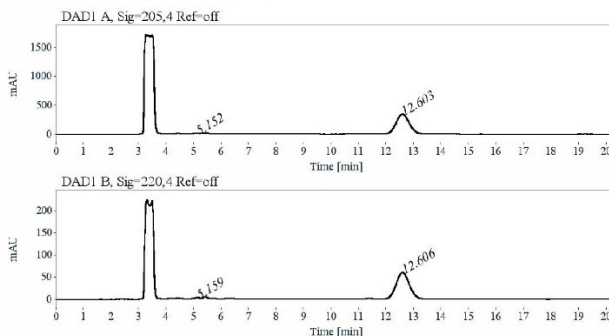
Signal: DAD1 B, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
5.19	7294	99.61	0.76		
12.63	29	0.39	3.28	4.33	16.10
Sum	7323	100.00			

## Z-Phe-D-Val-Leu<sub>2</sub>-OBn

### Chiral HPLC report

Sample name: YY600A  
Column: Chiralpak IE  
Temperature:  
Mobile phase: Heptane/Ethanol (50/50), 1 mL/min



Signal: DAD1 A, Sig=205,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
5.15	207	1.78	0.75		
12.60	11422	98.22	3.27	4.38	11.05
Sum	11629	100.00			

Signal: DAD1 B, Sig=220,4 Ref=off

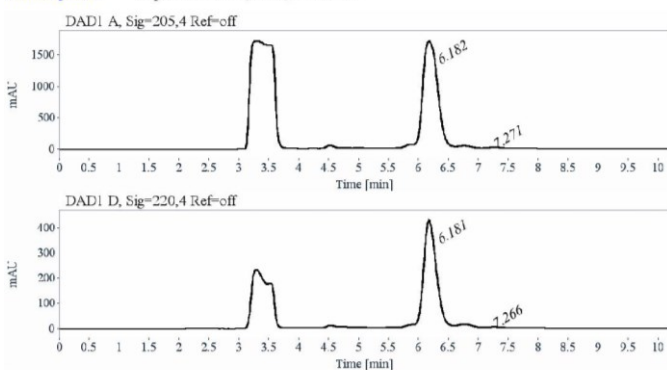
RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
5.16	40	1.92	0.75		
12.61	2023	98.08	3.27	4.37	10.63
Sum	2062	100.00			

Figure 11: Chromatograms of Z-Phe-Val-Leu<sub>2</sub>-OBn and Z-Phe-D-Val-Leu<sub>2</sub>-OBn

## Z-Phe-Val-Leu<sub>3</sub>-OBn

### Chiral HPLC report

Sample name: YY601A  
Column: Chiralpak IE  
Temperature:  
Mobile phase: Heptane/Ethanol (60/40), 1 mL/min



Signal: DAD1 A, Sig=205,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.18	28949	99.31	1.10		
7.27	201	0.69	1.46	1.34	2.78
Sum	29150	100.00			

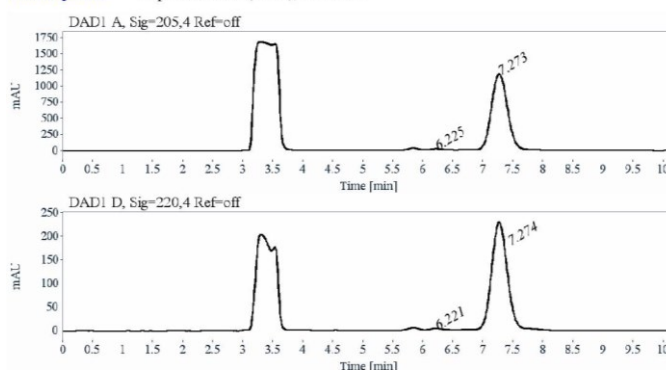
Signal: DAD1 D, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.18	6184	99.50	1.10		
7.27	31	0.50	1.46	1.34	2.96
Sum	6215	100.00			

## Z-Phe-D-Val-Leu<sub>3</sub>-OBn

### Chiral HPLC report

Sample name: YY602A  
Column: Chiralpak IE  
Temperature:  
Mobile phase: Heptane/Ethanol (60/40), 1 mL/min



Signal: DAD1 A, Sig=205,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.22	265	1.22	1.11		
7.27	21400	98.78	1.47	1.32	2.53
Sum	21665	100.00			

Signal: DAD1 D, Sig=220,4 Ref=off

RT [min]	Area	Area%	Capacity Factor	Enantioselectivity	Resolution (USP)
6.22	51	1.25	1.11		
7.27	3998	98.75	1.47	1.32	2.56
Sum	4049	100.00			

Figure 12: Chromatograms of Z-Phe-Val-Leu<sub>3</sub>-OBn and Z-Phe-D-Val-Leu<sub>3</sub>-OBn

## Abbreviations

DIC	<i>N,N'</i> -diisopropylcarbodiimide
EDC·HCl	<i>N</i> -(3-Dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide hydrochloride
HATU	<i>N</i> -[(Dimethylamino)-1 <i>H</i> -1,2,3-triazolo-[4,5- <i>b</i> ]pyridin-1-ylmethylene]- <i>N</i> -methylmethanaminium hexafluorophosphate <i>N</i> -oxide
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
HOBT·H <sub>2</sub> O	1-Hydroxybenzotriazole hydrate
Oxyma	Ethyl hydroxyiminocyanoacetate

## Author contributions

Y.Y. carried out the syntheses and the chemical analyses. Y.Y. and M.J. carried out the HPLC analyses aiming at measuring diastereomeric excesses. T.X.M. devised the presented idea, obtained the funding, supervised the project and wrote the manuscript. Y.Y., G.S., J.M., and F.L. discussed the results and commented on the manuscript.

## References

- (1) Friščić, T.; Childs, S. L.; Rizvi, S. A. A.; Jones, W. The role of solvent in mechanochemical and sonochemical cocrystal formation: a solubility-based approach for predicting cocrystallisation outcome. *CrystEngComm* **2009**, *11*, 418-426, DOI: 10.1039/B815174A.
- (2) Pietrancosta, N.; Quéléver, G.; Laras, Y.; Garino, C.; Burlet, S.; Kraus, J. L. Design of  $\beta$ -Secretase Inhibitors by Introduction of a Mandelyl Moiety in DAPT Analogues. *Aust. J. Chem.* **2005**, *58*, 585-594, DOI: 10.1071/CH05102.
- (3) Katritzky, A. R.; Suzuki, K.; Singh, S. K. Highly Diastereoselective Peptide Chain Extensions of Unprotected Amino Acids with *N*-(*Z*- $\alpha$ -Aminoacyl)benzotriazoles. *Synthesis* **2004**, *2004*, 2645-2652, DOI: 10.1055/s-2004-831255.
- (4) Yeboue, Y.; Gallard, B.; Le Moigne, N.; Jean, M.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide couplings by reactive extrusion: solid-tolerant and free from carcinogenic, mutagenic and reprotoxic chemicals. *ACS Sustainable Chem. Eng.* **2018**, *6*, 16001-16004, DOI: 10.1021/acssuschemeng.8b04509.
- (5) Popovic, S.; Bieräugel, H.; Detz, R. J.; Kluwer, A. M.; Koole, J. A. A.; Streefkerk, D. E.; Hiemstra, H.; van Maarseveen, J. H. Epimerization-Free C-Terminal Peptide Activation. *Chem. Eur. J.* **2013**, *19*, 16934-16937, DOI: 10.1002/chem.201303347.
- (6) Porte, V.; Thioly, M.; Pigoux, T.; Métro, T.-X.; Martinez, J.; Lamaty, F. Peptide Mechanochemistry by Direct Coupling of *N*-Protected  $\alpha$ -Amino Acids with Amino Esters. *Eur. J. Org. Chem.* **2016**, 3505-3508, DOI: 10.1002/ejoc.201600617.

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