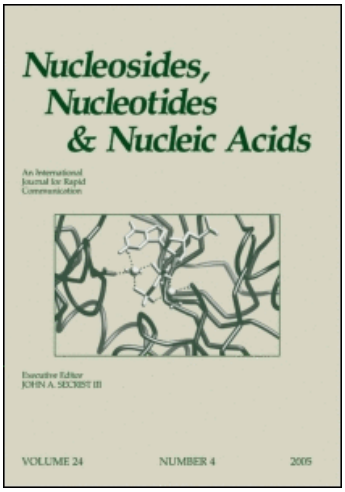

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Synthesis Of N-Labeled Peptidyl AMP

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SYNTHESIS OF *N*-LABELED PEPTIDYL AMP

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ABSTRACT: This paper deals with the synthesis of a new type of *N*-labeled peptidyl AMP, which would be used as a good substrate for analysis of the peptidyl transfer reaction on ribosome and for co-crystallization with ribosome. 4-(Dimethylamino)azobenzene-4'-sulfonyl (Dabsyl) was selected as the labeling group. (*N*-Dabsylglycyl)-L-leucyl AMP was synthesized from glycyl-L-leucine *via* a three-step procedure.

Introduction

Peptidyl transfer is a crucial step in protein biosynthesis on ribosome, where the peptidyl moiety of peptidyl-tRNA is transferred to the amino group of aminoacyl-tRNA, but its mechanism has not yet been clarified at the molecular level.[1-3] To understand the structure-function relationship of peptidyl transferase, many efforts to simplify the reaction have been made to date.[4-7] Fragment reaction,[4] *i. e.*, a simplified system has been used as a useful model of the peptidyl transferase-mediated reaction and requires

This paper is dedicated to the memory of the late Professor Alexander Krayevsky.

substrates such as radioisotope-labeled *N*-formylmethionyl oligonucleotides and puromycin as analogs of peptidyl-tRNA and aminoacyl-tRNA, respectively.

We have tried to assay the fragment reaction easily and quantitatively. For this purpose, we decided to synthesize non-radioisotope labeled aminoacyl- or peptidyl-tRNA analogs.

Several aminoacylated or peptidylated dinucleoside diphosphates[8] or dinucleoside monophosphates[9-11] have been chemically synthesized as substrates for the enzymatic synthesis of aminoacylated or peptidylated RNA oligomers. In these syntheses, the common strategy is to incorporate appropriately activated amino acid derivatives into the 2'- or 3'- hydroxyl group of the adenosine residue. The hitherto most useful activated amino acid species for this purpose have proved to be their cyanomethyl esters.[9-11]

We applied the cyanomethyl ester strategy to synthesize non-radioisotope labeled aminoacyl- or peptidyl- tRNA analogs. In the present study, AMP was used as the simplest partial structure of the 3'-terminal of aminoacyl tRNA and (*N*-dabsylglycyl)-L-leucine (**3**) as a non-radioisotope labeled peptide.[12] Information obtained from such a simple reaction system would be useful to synthesize other more complex derivatives such as non-radioisotope labeled aminoacyl dinucleoside diphosphates or aminoacyl dinucleoside monophosphates. In addition, such a new type of *N*-labeled peptidyl AMP would be also used for co-crystallization with ribosome.

Results and Discussion

Synthesis of (*N*-Dabsyl glycyl)leucine 3

First, the *N*-sulfonylation of the dipeptide **1** was carried out by the Schotten-Baumann reaction using dabsyl chloride (**2**).[13,14] For completion of this reaction, 1.5 equiv of dabsyl chloride was required. Since compounds having a dabsyl group can be visualized as brown-colored materials on TLC because of its inherent strong absorption around 460 nm, the *N*-dabsylation was easily monitored on TLC and isolation of the product was done with the help of this color marker. The *N*-dabsylated product **3** was obtained in 54% yield. Although the Schotten-Baumann reaction is effective for sulfonylation of amino acids, we have investigated a more improved procedure because of the rather low yield and the potential racemization problem caused by the excess **2** used in this procedure.

It is likely that dabsyl chloride reacts with not only the amino but also the carboxyl group, and an excess amount of moisture-sensitive dabsyl chloride must be required. Therefore, the *N*-dabsylation was carried out in an organic solvent to avoid considerable hydrolysis of the sulfonyl chloride. (Figure 1. Route A) Although the reaction of **1** with 2 equiv of **2** in pyridine gave the *N*-dabsylated product **3** in 83% yield as expected, it turned out that the leucine residue considerably racemized. The racemization was also detected even in the reaction of the cyanomethyl ester **4** with adenosine 5'-monophosphate bis(tetrabutylammonium) salt. Similar racemization of dipeptides during the activation by *p*-toluenesulfonyl chloride in the peptide synthesis was previously reported by Theodoropoulos.[15] The racemization might proceed via the oxazolinone intermediate[16] formed after the reaction of the sulfonyl chloride with the free carboxyl group.

It should be noted, however, that the racemization-free product (**L-3**) was successfully obtained *via* transient trimethylsilylation of the carboxyl group of **1** with 4 equiv of chlorotrimethylsilane in pyridine to avoid the oxazolinone formation. (Route B in Figure 1.)

Synthesis of (*N*-Dabsylglycyl)leucine Cyanomethyl Ester 4

The 2'-*O*- or 3'-*O*-aminoacylation of AMP has been performed by using appropriately activated amino acid derivatives.[17] However, the selective mono-aminoacylation of unprotected AMP was difficult in many cases because several side reactions such as *N*-acylation and bis-*O*-aminoacylation simultaneously occurred.[18] Schults *et al.* have reported the effective chemical aminoacylation of pdCpA by use of reactive cyanomethyl esters of *N*-protected amino acid derivatives.[9-11] To determine the best conditions to obtain the cyanomethyl ester, (*N*-dabsylglycyl)-DL-leucine was allowed to react with 3 equiv of chloroacetonitrile in the presence of 5 equiv of triethylamine in a manner similar to that described for the synthesis of amino acid cyanomethyl esters.[10] However, a considerable amount of a dialkylated product was formed along with the desired monoalkylated species. Replacement of triethylamine by a stronger organic base, 1,8-diazabicyclo[5,4,0]-7-undecene (DBU), gave a similar result. Finally, it was found that, when a lesser amount (1.0 equiv) of triethylamine was used in the presence of a large excess amount (40 equiv) of chloroacetonitrile, the mono-cyanomethylation proceeded predominantly to give the desired (*N*-Dabsylglycyl)-DL-leucine cyanomethyl ester (**DL-4**) which could be isolated in 58% yield by silica gel

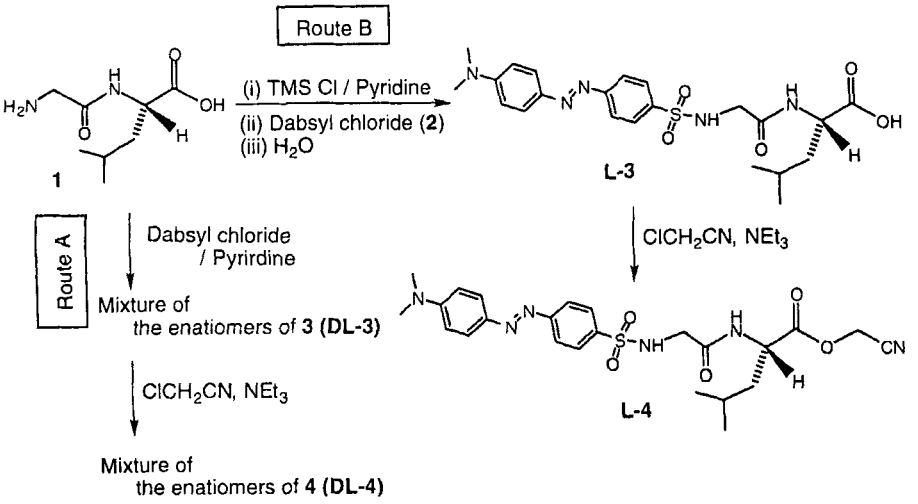

Figure 1. The synthetic route to obtain (*N*-dabsylglycyl)-L-leucine and its cyanomethyl ester.

column chromatography. The enantiomerically pure **L-4** was also obtained from **L-3** according to this procedure (Figure 1).

Synthesis of (*N*-Dabsyl Glycyl)leucyl AMP 5

First, the 2'-*O*- (3'-*O*-)peptidylation was performed by the reaction of 5'-AMP with the (*N*-dabsylglycyl)leucine cyanomethyl ester[19-22] obtained by Route A shown in Figure 1. Since the free acid of AMP is insoluble in organic solvents, this nucleotide has previously been used as a tetrabutylammonium salt that is more soluble in DMF. Schultz et al.[9] recommended the use of a 0.2 equiv excess amount of tetrabutylammonium hydroxide when the quaternary salt of pdCpA was prepared. Therefore, we attempted to prepare the tetrabutylammonium salt of AMP by using 1.2 equiv of tetrabutylammonium hydroxide for neutralization of the free AMP. However, the quaternary salt thus prepared failed to react with **4** in DMF. After extensive screening, it was found that the salt of AMP obtained by addition of 2 equiv of tetrabutylammonium hydroxide (AMP•2TBA) rapidly reacted with **4** to give the desired (*N*-dabsylglycyl)leucyl AMP (**5**) as the main product. The reaction was quenched after 20 min by addition of an ammonium acetate buffer (pH 4.5) to monitor the enantiomeric

purity of **4**. The reaction was quenched in such a short period because Schultz et al.[9] reported that the prolonged reaction time gave a small but detectable amount of racemized product during the aminoacylation of a dinucleoside diphosphate.

The structure and purity of **5** were examined by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of clearly showed the presence of the adenylic acid and (*N*-dabsylglycyl)leucine moieties of **5** as a mixture of the 2' or 3'-O-peptidylated regioisomers (1:4 as calculated from the 1'-H signals in the spectrum). In addition, the same spectrum also suggested the presence of a side product whose structure was quite similar to **5** as far as their ^{1}H NMR spectra were concerned. The side product was presumed to be the diastereoisomer of **5** having (*N*-dabsylglycyl)-D-leucyl instead of (*N*-dabsylglycyl)-L-leucyl based on the similarity of the ^{1}H NMR spectrum, the absence of any other spots and peaks in the TLC analysis, and MALDI-TOF mass spectra. The ratio of the two isomers was approximately 3:2 as determined by the ^{1}H NMR spectrum. The racemization of the L-leucine residue must occur during the sulfonylation by using 2 equiv of dabsyl chloride as described above. The racemization might proceed via the oxazolinone formation[16] following the reaction of the sulfonyl chloride with the free carboxyl group.

Therefore we synthesized **5** by using cyanomethyl ester **4** obtained in Route B (Figure 1, **L-4**). In this case, the content of racemized side products was reduced to the noise level of ^{1}H NMR and **5** was isolated in 35% yield. This result indicated that compound **4** synthesized in Route B was enantiomerically pure.

Interestingly, the desired **5** was effectively adsorbed on Dowex 50W-X8 resin that was used for the salt exchange when the organic layer extract was passed through the resin, while the unreacted species **4** and AMP•2TBA were eluted without adsorption. Thus, compound **5** was isolated with ease simply by elution with pyridine despite the low coupling efficiency. It should be emphasized that the isolation procedure of **5** is simple.

Next, we examined longer reaction time in order to improve the isolation yield of stereochemicaly pure **5**. An equimolar amount of AMP•2TBA was allowed to react with the enantiomerically pure **L-4** in DMF for various reaction periods, and the product **5** was isolated as described above. Although the isolation yield of **5** was improved up to 65% after 90 min, these conditions led to considerable racemization (ca 10 %). These results suggested that the leucine residue of **L-4** would racemize under

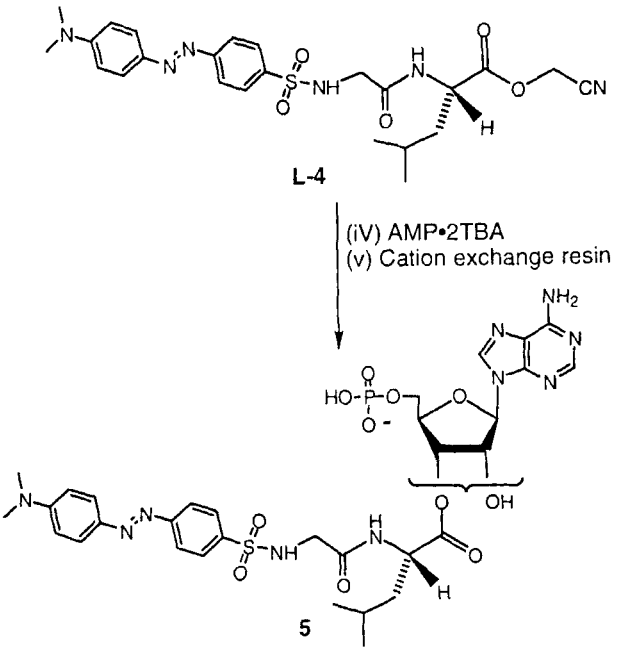

Figure 2. Reaction of the cyanomethyl ester **4** with AMP

the conditions used in the coupling reaction of AMP•2TBA and the cyanomethyl ester. Although a similar racemization was reported by Robertson, the racemization rate observed in the present study was higher than those reported by them.

Finally we found that the stereochemically pure **5** was obtained by using an excess amount of **L-4** (5 equiv). Interestingly , under these conditions prolonged reaction (90 min) did not induce racemization and gave the stereomerically pure **5** in 41% yield.

A mixture of the 2' or 3'-*O*-peptidylated regioisomers obtained in this study can be used in biochemical assays, because the 2' and 3'-*O* peptidyl group migrate from one to the other rapidly in aqueous solution even under neutral pH.[23]

The UV spectrum of **5** is shown in Figure 3. The λ_{max} peak at 480 nm has intensity similar to that observed 255 nm so that compound **5** can be easily detected without a UV detector. Further studies are now under way directed toward application of compound **5** as a visually detectable peptidyl tRNA analog to the analysis of the mechanism of aminoacyl transfer reaction in ribosome as well as the co-crystallization with ribosome.

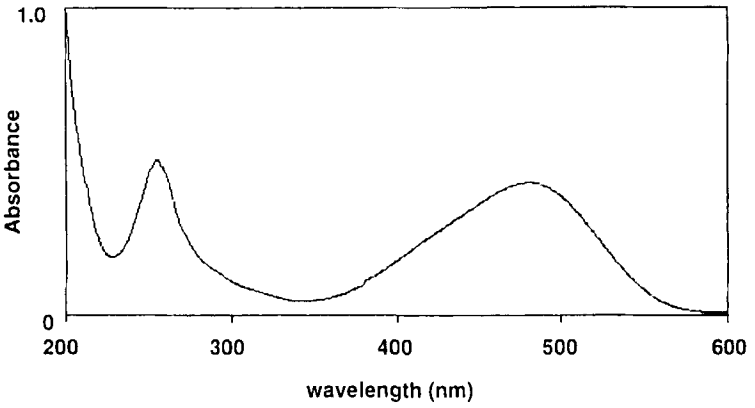

Figure 3. Absorption Spectrum of *N*-Dabsyl Glycyl-L-leucyl AMP (**5**)

Experimental

DMF and acetonitrile were distilled from CaH_2 after being refluxed for several hours and stored over 4A molecular sieves. Pyridine was distilled after being refluxed over *p*-toluenesulfonyl chloride for several hours, redistilled from CaH_2, and stored over 4A molecular sieves. 4-(Dimethylamino)azobenzene-4'-sulfonyl chloride (dabsyl chloride), chloroacetonitrile, glycyl-L-leucine and 10% tetrabutylammonium hydroxide in methanol were purchased from Tokyo Kasei Kogyo Co. Ltd. (Japan). Adenosine 5'-monophosphate (AMP) was purchased from Sigma-Aldrich Co. Ltd. Thin layer chromatography (TLC) was performed on silica gel (Merck, 60 F-254) in the following solvent systems: (A) chloroform-methanol-acetic acid (95:5:1, v/v/v), (B) hexane-ethyl acetate (1:1, v/v), (C) butanol-acetic acid-water (5:2:3, v/v/v). ^1H and ^{13}C NMR spectra were recorded at 270 and 67.8 MHz, respectively, on JEOL-EX 270 using tetramethylsilane (TMS) as an internal standard. UV spectra were obtained on a HITACHI U2000 spectrophotometer. MALDI-TOF mass spectra were obtained on a Voyager RP spectrometer.

(*N*-Dabsylglycyl)-L-leucine (3). Schotten-Baumann reaction: Glycyl-L-leucine (188 mg, 1 mmol) was dissolved in 0.25 M NaOH (4 ml). To this solution was added a DMF solution (4 ml) of dabsyl chloride (486 mg, 1.5mmol). The mixture was stirred at room temperature for one day while the pH value was monitored and a portion of 0.25 M NaOH was added so as to maintain pH 10. 1 M HCl was added to neutralize

the solution. The solution was extracted three times with chloroform, and all the organic solution was collected, dried over $NaSO_4$, filtered off, and concentrated to a small volume under reduced pressure. The residue was purified by chromatography on a silica gel column using an stepwise gradient of methanol in 1% increment (0-10%) in chloroform to give **3** (260 mg) in 54% yield.

Route A: A mixture of glycyl-L-leucine (188 mg, 1 mmol) and dabsyl chloride (647 mg, 2 mmol) was dissolved in dry pyridine (10 ml). The mixture was stirred at room temperature for three days. The solvent was evaporated, and the residue was partitioned between chloroform (50 ml) and water (50 ml). The organic layer was washed twice with water (50 ml), dried over anhydrous sodium sulfate, and concentrated to a small volume under reduced pressure. The product was purified by chromatography on a silica gel column using a stepwise gradient of methanol in 1% increment (0-10%) in chloroform to give **DL-3** . The total amount of the mixture was 395 mg (83% yield).

Route B: Glycyl-L-leucine (4.0 g, 22 mmol) and chlorotrimethylsilane (11 ml, 86 mmol) were dissolved in pyridine (220 ml), and the mixture was stirred at room temperature for 20 min. Dabsyl chloride (7.0 g, 22 mmol) was added portionwise, and the resulting solution was stirred at room temperature. After 2 h, the solution was poured into pyridine-water (150 ml-150 ml), and the solvent was evaporated under reduced pressure. The residue was extracted with chloroform (300 ml x 2). The organic layer was dried over anhydrous magnesium sulfate, and concentrated to a small volume under reduced pressure. The product was purified by chromatography on a silica gel column using a stepwise gradient of methanol in 1% increment (0-10%) in chloroform to give **L-3** (10.5 g, 82%): UV (CH_2Cl_2) λ_{max} 447 nm, 272 nm, λ_{min} 340 nm; ^{1}H NMR (DMSO-d6) δ 0.75(3H, d, J = 5.6 Hz, CH_3 of Leu), 0.81 (3H, d, J = 5.6 Hz, CH_3 of Leu), 1.42 (3H, m, CH and CH_2 of Leu), 3.08 (6H, s, CH_3 of Dbs), 3.54 (2H, q, CH_2 of Gly), 4.11 (1H, m, α-H of Leu), 6.84 (2H, d, J = 8.6 Hz, ArH), 7.80-7.88-8.09 (8H, m, ArH, NH), 12.59 (1H, br, COOH); ^{13}C NMR (DMSO-d6) δ21.30, 22.73, 24.10, 44.86, 50.10, 111.57, 122.03, 125,34, 127.91, 140.11, 142.61, 153.08, 154.47, 167.30, 173.71; MALDI-TOF mass Calcd for *m/z* $C_{22}H_{30}N_5O_5S$ $[M+H]^+$ 476.20. Observed for *m/z* 476.56.

Typical Procedure for the (*N*-Dabsylglycyl)leucine Cyanomethyl Ester. (*N*-Dabsylglycyl)-DL-leucine (**DL-3**) (888 mg, 1.867 mmol) was dissolved in dry

acetonitrile (20 ml). Distilled triethylamine (279 μl, 2 mmol) and chloroacetonitrile (5.03 ml, 80 mmol) were added stepwise to the solution. The reaction mixture was stirred at room temperature for one day. The solvent was evaporated under reduced pressure, and the residue was partitioned between chloroform (50 ml) and water (50 ml). The organic layer was washed twice with water (50 ml), dried over anhydrous sodium sulfate, and concentrated to a small volume under reduced pressure. The product was purified by chromatography on a silica gel column using a stepwise gradient of ethyl acetate in 10% increment (0-70%) in hexane to give the cyanomethyl ester **DL-4** (556 mg) in 58% yield: R_f(B) = 0.24; UV (dioxane) λ_{max} 433 nm, 268 nm, λ_{min} 336 nm, 226 nm; ^{1}H NMR ($CDCl_3$) δ 0.93 (6H, d, J = 6.6 Hz, CH_3 of Leu), 1.64 (3H, m, CH and CH_2 of Leu), 3.12 (6H, s, CH_3 of Dbs), 3.68 (2H, t, J = 6.3 Hz, CH_2 of Gly), 4.61 (1H, m, α-H of Leu), 4.74 (2H, q, CH_2CN), 5.48 (1H, t, NH), 6.65 (1H, d, NH), 6.75 (2H, d, J = 9.2 Hz, ArH), 7.90 (2H, d, J = 9.2 Hz, ArH), 7.93 (4H, m, ArH); ^{13}C NMR ($CDCl_3$) δ 21.55 (CH_3 of Leu), 22.72 (CH_3 of Leu), 24.73 (CH of Leu), 40.27 (NCH_3), 40.48 (CH_2 of Leu), 45.66 (α-C of Gly), 49.02 (CH_2CN), 50.59 (α-C of Leu), 111.45 (3,5-C), 113.95 (CN), 122.82, 125.84, 128.23, 137.74 (4-C), 143.56 (4'-C), 153.21 (1-C), 156.03 (1'-C), 168.14 (C(O)NH), 171.19 (C(O)O); MALDI-TOF mass Calcd for *m/z* $C_{24}H_{31}N_6O_5S$ $[M+H]^+$ 515.21. Observed for *m/z* 515.02.

Enantiomerically pure (*N*-Dabsyl glycyl)-L-leucine cyanomethyl ester (**L-4**) was obtained from **L-3** (1.1 g, 2.3 mmol), triethylamine (350 μl, 2.5 mmol) and chloroacetonitrile (6.3 ml, 98 mmol) according to the above procedure. The yield was 875 mg (74% yield): ^{1}H NMR ($CDCl_3$) δ 0.92 (6H, dd, J = 6.3 Hz, CH_3 of Leu), 1.62 (3H, m, CH and CH_2 of Leu), 3.12 (6H, s, CH_3 of Dbs), 3.68 (2H, t, J = 5.9 Hz, CH_2 of Gly), 4.61 (1H, m, α-H of Leu), 4.75 (2H, q, CH_2CN), 6.75 (2H, d, J = 9.2 Hz, ArH), 7.90 (2H, d, J = 9.2 Hz, ArH), 7.93 (4H, m, ArH).

General Procedure for (*N*-Dabsylglycyl)leucyl AMP. AMP (1 g, 2.74 mmol, free form) was neutralized with 2 equiv of tetrabutylammonium hydroxide (10% solution) in methanol. This solution was evaporated under reduced pressure and freeze-dried to give white foam. The AMP•2TBA thus obtained can be stocked for months at –20 °C. To a 0.1 M solution of AMP•2TBA in DMF was added the 1.0-5.0 equiv of (*N*-dabsyl glycyl)leucine cyanomethyl ester (**DL- or L-4**). The reaction mixture was stirred at room temperature for 20-90 min. The reatcion mixture was poured into 50 mM ammonium acetate buffer (pH 4.5, 20 ml), and the resulting mixture was extracted with

chloroform (30 mlx2). To the organic layer was added a Dowex 50W-X8 (pyridinium form, 20ml / 0.36 mmol of AMP) to exchange the cation form. The resin was washed with chloroform (50 ml). Elution with pyridine (50 ml) followed by evaporation gave **5** or their diastereomeric mixture.

(*N*-Dabsylglycyl)-L-leucyl AMP 5. Thitle compound was synthesized according to the above genteral procedure. The reaction of AMP•TBA (300 mg, 0.36 mmol) with (*N*-dabsyl glycyl)-L-leucine cyanomethyl ester (**L-4**) (926 mg, 1.8 mmol) for 90 min gave **5** (119.8 mg, 41%): UV (H_2O) λ_{max} 481 nm, 258 nm, λ_{min} 343 nm, 228 nm; ^1H NMR (DMSO-d6) 3' regioisomer: δ 0.72 (3H, d, J = 5.6 Hz, CH_3 of Leu), 0.79 (3H, d, J = 5.6 Hz, CH_3 of Leu), 1.46 (3H, m, CH amd CH_2 of Leu), 3.02 (6H, s, CH_3 of Dbs), 3.50-3.60 (2H, m, C(O)CH_2 of Gly), 4.05-4.27 (2H, m, 4'-H, C(O)CH of Leu), 4.78 (1H, dd, J = 6.3 Hz, 2'-H), 5.23 (1H, m, 3'-H), 5.89 (1H, d, J = 7.3 Hz, 1'-H), 6.79 (2H, d, J = 9.2 Hz, ArH), 7.74-7.89 (6H, m, ArH + NH), 8.15 (1H, s, 2-H or 8-H), 8.34 (1H, s, 2-H or 8-H). 2' regioisomer : δ 0.64 (d, CH_3 of Leu), 4.52 (m), 5.50 (t, 2'-H), 6.08 (d, 1'-H); ^{13}C NMR (DMSO-d6) mixture of 3' and 2' regioisomer δ 21.31, 22.66, 24.05, 44.84, 50.25, 64.94, 71.86, 73.53, 80.74, 86.42, 111.54, 118.83, 122.03, 123.90, 125.32, 125.79, 127.87, 136.26, 139.00, 140.04, 142.63, 149.36, 149.63, 152.53, 153.07, 154.50, 155.89, 167.58, 171.12. ; ^{31}P NMR (DMSO-d6) δ -0.09; MALDI-TOF mass Calcd for m/z $C_{32}H_{40}N_{10}O_{11}PS$ $[M+H]^+$ 805.25. Observed for m/z 805.35.

Acknowledgements

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