Synthesis of Trehalose-centered Dipeptide Esters

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Chemical esterification of α,α-trehalose with two different dipeptide acyl donors, Boc-Phe-Met-OH and Boc-Gly-Gly-OH, gave a set of products differing in the number and site of substitution. With both dipeptides 6,6'-diester was isolated as the major product. In the Boc-Phe-Met-OH line, the dipeptide units in all higher substituted esters were asymmetrically distributed between the two trehalose rings. In contrast, the first higher substituted Boc-Gly-Gly-OH derived ester found was the symmetric 2,2',6,6'-tetraester. These four sites remained occupied in all other isolated products, two asymmetric pentaesters and one symmetric hexaester. The data presented here show that in addition to sugar hydroxyl group reactivity, the structural properties of the acylating reagent, in particular its sterical demands, are of the utmost importance for the distribution and arrangement of acyl moieties around the non-reducing disaccharide core molecule.

INTRODUCTION

Peptides of various size as well as peptide mimetics will comprise a large part of future therapeutics. Promising lead candidates are already being discovered by modification of natural products, phage display and combinatorial chemistry, and several of them are in clinical trials.[1–3] Owing to their small size, peptides are rapidly removed from the circulation by enzymatic degradation and by rapid filtration in the kidney. Various strategies for improving peptide half-lives *in vivo* are currently under investigation, including amino acid backbone modifications, formulation approaches, chemical conjugation with small molecules, enzyme inhibitors and absorption enhancers.[4] Control of peptide drug release can bring a number of benefits, including increased pharmacological efficacy, extended duration of action, greater flexibility of administrative route and improved targeting of specific disease sites.

Since carbohydrates play an important role in the cell function of multicellular organisms and in biological molecular recognition, one of the main objectives in glycobiology is the investigation of carbohydrate-centered dendrimers as tools for efficient multivalent presentation of biological ligands in biospecific recognition, inhibition and targeting.[5–8] Sugars provide abundant reactive OH groups that can be utilized to attach bioactive peptides through a simple reaction such as esterification. It can be expected that conjugation of a peptide with a carbohydrate molecule will provide a convenient matrix for stabilization, targeting and controlled release of the parent peptide compound. Despite the ready construction of ester-carbohydrate linkages, there are few reports on enzymatic or chemical syntheses of sugar amino acid es-

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ters.[9–12] This is all the more surprising given that several carbohydrate-peptide esters have altered bioactivity[13] or high potential utility as prodrugs, in which the sugar moiety allows for simultaneous masking of an amino and a carboxyl group of a peptide.[14,15]

The specific properties and physiological functions of trehalose, a non-reducing disaccharide of glucose, and the expanding trehalose market, prompted chemical and enzymatic syntheses of trehalose derivatives. This sugar is widespread throughout the biological world, being found in organisms such as bacteria, fungi, yeasts, insects, plants, and many others.[16] Besides the essential role of trehalose in the protection of proteins and membranes from denaturation caused by different stresses, it was also shown that 2,3-diesters of trehalose may play an important role in determining the outcome of infection with tubercle bacillus.[17] Moreover, it was shown recently that trehalose, when given orally, can reduce the polygluta-mine-mediated insoluble protein aggregation in a mouse model of Huntington's disease.[18] In addition, trehalose derivatives with guanidino groups attached, are found to inhibit the Tat-TAR RNA interaction in human cells, which is essential for HIV-1 replication.[19] Taking into account all the newly discovered potential benefits of trehalose derivatives, along with the previously known but still not completely clear role of trehalose in signaling pathways and membrane-protecting mechanisms, there are many issues to be addressed within this field. One of the possible approaches is to uncover the susceptibility of the trehalose active sites (hydroxyl groups) towards certain reactions or distinct substituents. While enzymatic synthesis of sugar amino acid esters can disclose the acyl donor and acyl acceptor specificity of certain enzymes,[9,11,20] insight into the chemical reactivity of particular sugar hydroxyl groups can be gained only by chemically performed esterification.

Following this issue, by using trehalose as a multivalent core molecule, in this paper we investigate the products formed by chemical esterification of the disaccharide acceptor with two different dipeptides. As acyl donors, Boc-Phe-Met-OH and Boc-Gly-Gly-OH were chosen to probe how two structurally very different N-terminally protected dipeptides will compete for the esterification of the trehalose hydroxyl groups.

EXPERIMENTAL

General Methods

Melting points were determined on a Tottoli (Büchi) apparatus and were uncorrected. Reactions were monitored by TLC on Silica Gel 60 F$_{254}$ plates (Merck; Darmstadt, Germany) applying detection with ninhydrin, chlorine-iodine reagent or heating with H$_2$SO$_4$.

NMR spectra were recorded on a Bruker AV 600 spectrometer operating at 75.47 MHz for ^{13}C and 600.13 MHz

for ^{1}H. Spectra were assigned on the basis of 2D homonuclear (COSY) and heteronuclear (HMQC) spectra.

Mass spectra were recorded on a ThermoFinnigan LCQ Deca ion trap mass spectrometer, operating in Electrospray Ionization (ESI) mode. The ion source and ion optics of the instrument were tuned for optimal sensitivity using the autotune function of the instrument's Xcalibur (v. 1.3) software, by infusing a 50 μg/ml solution of compound in methanol into the ion source of the instrument from a syringe pump at a flow rate of 10 μl/min.

Full scan ESI-MS spectra were recorded in the m/z range of 150–2300, while the m/z ranges for MS/MS and MS^3 spectra were selected according to the m/z value of the precursor ions. MS/MS spectra were recorded at both low and high collision energy settings, the low setting being 25 and the high being 80 units (the units are dimensionless because they are defined by the software).

Chemicals

General procedure for the synthesis of trehalose-derived dipeptide esters: α,α-trehalose (0.1 mmol) was dissolved in dry pyridine and treated with the pentachlorophenyl ester of N-*tert*-butyloxycarbonyl-L-phenylalanyl-L-methionine (Boc-Phe-Met-OPCP) or N-*tert*-butyloxycarbonyl-glycylglycine (Boc-Gly-Gly-OPCP) (1 mmol) in the presence of imidazole (5 mmol). The reaction mixture was stirred at room temperature for 24 hours. The solvent was evaporated *in vacuo* and the residue was purified by flash chromatography on silica gel with chloroform-methanol-acetic acid (10:3:0.3) as the eluent. The fractions enriched with trehalose esters were collected and further purified by column chromatography with toluene-ethanol-ethyl acetate (4:2:1) as the eluent.

6,6′-Di-O-(Boc-Phe-Met-)-α,α-trehalose (1)

^{13}C NMR (DMSO-d_6) δ/ppm 14.43 (S-CH$_3$, Met), 28.08 (CH$_3$, Boc), 29.39 (Cγ, Met), 30.61 (Cβ, Met), 37.11 (Cβ, Phe), 50.76 (Cα, Met), 55.52 (Cα, Phe), 78.00 (C, Boc), 126.13 (Cζ, Phe), 128.15 (Cε, Phe), 129.16 (Cδ, Phe), 138.14 (Cγ, Phe), 155.24 (CO, Boc), 171.57, 172.17 (CO, Phe, Met). ^{1}H NMR (DMSO-d_6) δ/ppm 1.27 (18H, CH$_3$, Boc), 1.87/1.97 (4H, β,β', Met), 2.04 (6H, S-CH$_3$, Met), 2.43/2.53 (4H, γ,γ' Met), 2.71/2.94 (4H, β,β', Phe), 4.16 (2H, α, Phe), 4.44 (2H, α, Met), 6.93 (2H, NH, Phe), 7.17–7.30 (10H, arom. H, Phe), 8.32 (2H, NH, Met). ^{1}H and ^{13}C NMR data for the trehalose moiety are presented in Tables I and II. M.p. 125–131 °C; ESI-MS Calcd. for C$_{50}$H$_{74}$N$_4$O$_{19}$S$_2$ 1099.29. Found: (M+Na)$^+$ m/z 1121.2.

2,6,6′-Tri-O-(Boc-Phe-Met-)-α,α-trehalose (2)

^{13}C NMR (DMSO-d_6) δ/ppm 14.79, 14.97 (S-CH$_3$, Met), 28.29, 28.57 (CH$_3$, Boc), 29.68, 29.82, 29.88, 29.94 (Cβ, Cγ, Met), 37.53, 37.60 (Cβ, Phe), 51.28, 51.51, 52.01 (Cα, Met), 55.99 (Cα, Phe), 78.46, 78.78 (C, Boc), 126.59, 126.65 (Cζ, Phe), 128.45 (Cε, Phe), 129.66 (Cδ, Phe), 138.33, 138.65 (Cγ, Phe), 155.51, 155.73 (CO, Boc), 171.90, 171.94, 172.02, 172.17, 172.55, 172.67 (CO, Phe, Met). ^{1}H NMR (DMSO-d_6) δ/ppm 1.27 (27H, CH$_3$, Boc), 1.91/2.02, 1.92/2.16 (6H, β,β', Met), 2.04, 2.05 (9H, S-CH$_3$, Met), 2.32/2.40, 2.47/2.55

(6H, γ,γ' Met), 2.76/2.96 (6H, β,β', Phe), 4.21 (3H, α, Phe), 4.49 (3H, α, Met), 6.81 (3H, NH, Phe), 7.18–7.26 (15H, arom. H, Phe), 8.24 (3H, NH, Met). ^{1}H and ^{13}C NMR data for the trehalose moiety are presented in Tables I and II. M.p. 105–115 °C; ESI-MS Calcd. for $C_{69}H_{100}N_6O_{23}S_3$ 1477.78. Found: (M+Na)$^+$ *m/z* 1499.2.

Tri-O-(Boc-Phe-Met-)-α,α-trehalose (3)

^{13}C NMR (DMSO-d_6) δ/ppm 14.29, 14.43, 14.57 (S-CH$_3$, Met), 28.03 (CH$_3$, Boc), 29.21, 30.65 (Cβ, Cγ, Met), 37.14 (Cβ, Phe), 50.67, 50.80 (Cα, Met), 55.41 (Cα, Phe), 77.97 (C, Boc), 126.05 (Cζ, Phe), 127.89 (Cε, Phe), 129.11 (Cδ, Phe), 137.77, 138.06 (Cγ, Phe), 155.14, (CO, Boc), 171.42, 171.98 (CO, Phe, Met). ^{1}H NMR (DMSO-d_6) δ/ppm 1.29 (27H, CH$_3$, Boc), 1.82/1.90, 1.90/2.01 (6H, β,β', Met), 2.02, 2.04 (9H, S-CH$_3$, Met), 2.30/2.39, 2.45/2.54 (6H, γ,γ' Met), 2.76/2.96 (6H, β,β', Phe), 4.20 (3H, α, Phe), 4.44 (3H, α, Met), 6.80 (3H, NH, Phe), 7.19–7.25 (15H, arom. H, Phe), 8.23 (3H, NH, Met). M.p. 100–108 °C; ESI-MS Calcd. for $C_{69}H_{100}N_6O_{23}S_3$ 1477.78. Found: (M+Na)$^+$ *m/z* 1499.2.

Tetra-O-(Boc-Phe-Met-)-α,α-trehalose (4)

^{13}C NMR (DMSO-d_6) δ/ppm 14.31, 14.46, (S-CH$_3$, Met), 27.77, 28.06 (CH$_3$, Boc), 29.24, 30.55, 30.87, 31.25 (Cβ, Cγ, Met), 37.14 (Cβ, Phe), 50.33, 50.57, 50.82, 51.05 (Cα, Met), 55.44 (Cα, Phe), 63.10, 63.56 (C6,6',Tre), 77.99 (C, Boc), 90.51, 91.15, 91.36, 91.43, 93.44, 94.05, 94.35, 94.42, 94.71 (C1,1', Tre), 126.10 (Cζ, Phe), 127.92 (Cε, Phe), 129.13 (Cδ, Phe), 137.78, 138.11 (Cγ, Phe), 154.96, 155.18, 156.56 (CO, Boc), 170.98, 171.33, 171.44, 171.71, 171.95, 172.11 (CO, Phe, Met). ^{1}H NMR (DMSO-d_6) δ/ppm 1.29 (36H, CH$_3$, Boc), 1.8–2.1 (8H, β,β', Met), 2.0–2.07 (12H, S-CH$_3$, Met), 2.4–2.6 (8H, γ,γ' Met), 2.72/2.96 (8H, β,β', Phe), 3.55, 3.75, 3.85 (5H, Tre), 4.23 (4H, α, Phe), 4.47 (4H, α, Met), 4.5–5.6 (9H, Tre), 6.8–7.1 (4H, NH, Phe), 7.18–7.28 (20H, arom. H, Phe), 8.35 (4H, NH, Met). M.p. 107–115 °C; ESI-MS Calcd. for $C_{88}H_{126}N_8O_{27}S_4$ 1856.27. Found: (M+Na)$^+$ *m/z* 1877.4.

Penta-O-(Boc-Phe-Met-)-α,α-trehalose (5)

^{13}C NMR (DMSO-d_6) δ/ppm 14.33, 14.49, 14.64 (S-CH$_3$, Met), 27.84, 28.13, (CH$_3$, Boc), 29.25, 29.41, 30.64 (Cβ, Cγ, Met), 37.15 (Cβ, Phe), 50.62, 50.57, 51.02, 51.58 (Cα, Met), 55.56, 55.71 (Cα, Phe), 78.05 (C, Boc), 93.52, 94.04 (C1,1', Tre), 126.17 (Cζ, Phe), 128.01 (Cε, Phe), 129.21 (Cδ, Phe), 137.89, 138.20 (Cγ, Phe), 155.06, 155.28 (CO, Boc), 171.80 (CO, Phe, Met). ^{1}H NMR (DMSO-d_6) δ/ppm 1.29 (45H, CH$_3$, Boc), 1.8–2.1 (10H, β,β', Met), 2.0–2.07 (15H, S-CH$_3$, Met), 2.3–2.6 (10H, γ,γ' Met), 2.75/2.96 (10H, β,β', Phe), 3.15, 3.33, 3.52, 3.62, 3.80, 3.94, 4.10 (H, Tre), 4.19 (5H, α, Phe), 4.47 (5H, α, Met), 6.7–6.9 (5H, NH, Phe), 7.18–7.26 (25H, arom. H, Phe), 7.9–8.3 (5H, NH, Met). M.p. 105–113 °C; ESI-MS Calcd. for $C_{107}H_{152}N_{10}O_{31}S_5$ 2234.76. Found: (M+Na)$^+$ *m/z* 2256.8.

6,6'-Di-O-(Boc-Gly-Gly-)-α,α-trehalose (6)

^{13}C NMR (DMSO-d_6) δ/ppm 28.14 (CH$_3$, Boc), 40.42, 43.00 (Cα, Gly^1, Gly^2), 77.97 (C, Boc), 155.70 (CO, Boc),

177.18 (CO, Gly^1, Gly^2); ^{1}H NMR (DMSO-d_6) δ/ppm 1.38 (18H, CH$_3$, Boc), 3.35, 3.52, 3.58, 3.84 (8H, αα, Gly^1, Gly^2) 7.05, 8.36 (4H, NH, Gly^1, Gly^2). ^{1}H and ^{13}C NMR data for the trehalose moiety are presented in Tables I and II. M.p. 175–185 °C; ESI-MS Calcd. for $C_{30}H_{50}N_4O_{19}$ 770.31. Found: (M+Na)$^+$ *m/z* 793.0, (M–H)$^-$ *m/z* 769.2.

2,2',6,6'-Tetra-O-(Boc-Gly-Gly-)-α,α-trehalose (7)

^{13}C NMR (DMSO-d_6) δ/ppm 28.12 (CH$_3$, Boc), 40.39, 42.98 (Cα, Gly^1, Gly^2), 78.00 (C, Boc), 155.67 (CO, Boc), 169.38, 169.61, 169.67, 169.86 (CO, Gly^1, Gly^2); ^{1}H NMR (DMSO-d_6) δ/ppm 1.38 (36H, CH$_3$, Boc), 3.58, 3.85, 3.99 (16H, αα, Gly^1, Gly^2) 7.02, 8.18 (8H, NH, Gly^1, Gly^2). ^{1}H and ^{13}C NMR data for the trehalose moiety are presented in Tables I and II. M.p. 127–135 °C; ESI-MS Calcd. for $C_{48}H_{78}N_8O_{27}$ 1198.50. Found: (M+Na)$^+$ *m/z* 1221.5, (M–H)$^-$ *m/z* 1197.2.

2,2',3,6,6'-Penta-O-(Boc-Gly-Gly-)-α,α-trehalose (8)

^{13}C NMR (DMSO-d_6) δ/ppm 28.15 (CH$_3$, Boc), 40.29, 43.01 (Cα, Gly^1, Gly^2), 78.06, 78.09 (C, Boc), 155.73 (CO, Boc), 169.62, 169.66, 170.20, 170.26 (CO, Gly^1, Gly^2); ^{1}H NMR (DMSO-d_6) δ/ppm 1.37 (45H, CH$_3$, Boc), 3.60, 3.86 (20H, αα, Gly^1, Gly^2) 6.85, 7.00, 8.20 (10H, NH, Gly^1, Gly^2). ^{1}H and ^{13}C NMR data for the trehalose moiety are presented in Tables I and II. M.p. 120–128 °C; ESI-MS Calcd. for $C_{57}H_{92}N_{10}O_{31}$ 1412.59. Found: (M+Na)$^+$ *m/z* 1435.5, (M–H)$^-$ *m/z* 1411.2.

2,2',4,6,6'-Penta-O-(Boc-Gly-Gly-)-α,α-trehalose (9)

^{13}C NMR (DMSO-d_6) δ/ppm 28.19 (CH$_3$, Boc), 43.02 (Cα, Gly^1, Gly^2), 78.09, 78.19 (C, Boc), 155.77 (CO, Boc), 169.33, 169.49, 170.04, 170.32 (CO, Gly^1, Gly^2), 90.69, 91.09, 91.79 (C1,1', Tre), 72.48, 72.53, 72.80, 72.84 (C2,2', Tre), 67.12, 67.28, 69.80, 69.85, 69.92, 69.97, 70.29, 73.24, 73.50 (C3,3',4,4',5,5', Tre), 62.95, 63.47 (C6,6', Tre); ^{1}H NMR (DMSO-d_6) δ/ppm 1.38 (45H, CH$_3$, Boc), 3.60, 3.80, 3.85 (20H, αα, Gly^1, Gly^2), 4.20, 4.25 (4H, H6,6', Tre), 4.63, 4.86 (2H, H2,2', Tre), 3.73–5.50 (6H, H3,3',4,4',5,5', Tre), 5.01, 5.09 (2H, H1,1', Tre), 6.65, 7.00, 8.20 (10H, NH, Gly^1, Gly^2). M.p. 115–123 °C; ESI-MS Calcd. for $C_{57}H_{92}N_{10}O_{31}$ 1412.59. Found: (M+Na)$^+$ *m/z* 1435.5, (M–H)$^-$ *m/z* 1411.2.

2,2',4,4,'6,6'-Hexa-O-(Boc-Gly-Gly-)-α,α-trehalose (10)

^{13}C NMR (DMSO-d_6) δ/ppm 28.15 (CH$_3$, Boc), 40.29, 43.02 (Cα, Gly^1, Gly^2), 78.07 (C, Boc), 155.73 (CO, Boc), 169.42, 169.67, 169.97 (CO, Gly^1, Gly^2); ^{1}H NMR (DMSO-d_6) δ/ppm 1.37 (54H, CH$_3$, Boc), 3.56, 3.85, 3.95 (24H, αα, Gly^1, Gly^2) 6.99, 8.19 (12H, NH, Gly^1, Gly^2). ^{1}H and ^{13}C NMR data for the trehalose moiety are presented in Tables I and II. M.p. 145–155 °C; ESI-MS Calcd. for $C_{66}H_{106}N_{12}O_{35}$ 1626.69. Found: (M+Na)$^+$ *m/z* 1649.5, (M–H)$^-$ *m/z* 1625.3.

RESULTS AND DISCUSSION

Synthesis

As the starting carbohydrate material we utilized the symmetrical non-reducing disaccharide, α,α-trehalose, which
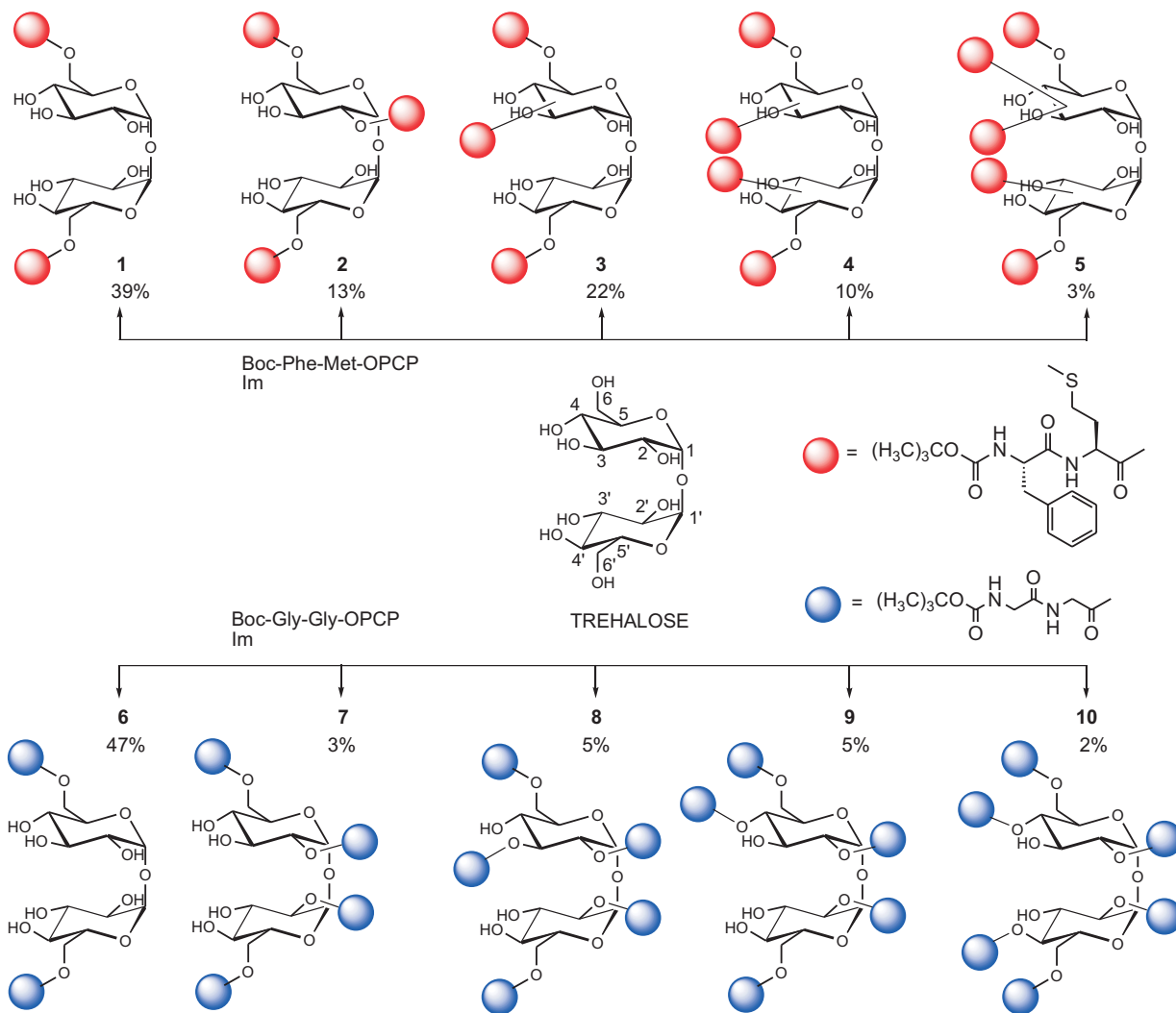
Scheme 1.

can be considered an anomerically protected D-glucose. Esterification reactions were studied with either *N-tert*-butyloxycarbonyl-L-phenylalanyl-L-methionine (Boc-Phe-Met-OH) or *N-tert*-butyloxycarbonyl-glycylglycine (Boc-Gly-Gly-OH) by treating trehalose with the corresponding dipeptide pentachlorophenyl ester, in the presence of imidazole as the promoting agent for transfer of the acyl group to the sugar component.[12,21] Pentachlorophenyl esters were chosen because a good leaving group at the acyl donor is essential for efficient esterification,[11] and this strategy should also allow comparison with previously published work on the synthesis of monosaccharide esters.[12] The molar proportions of the reactants used were sugar:dipeptide:imidazole, 1:10:5, in all reactions performed. The degree of substitution at the trehalose moiety was shown to be very sensitive to the structure of the starting dipeptide used in the esterification reaction.

In contrast to the regioselectivity observed previously in the reaction of Boc-Phe-Leu-OPCP with unprotected D-glucose,[22] the esterification of trehalose with the acti-vated dipeptide ester, Boc-Phe-Met-OPCP, afforded ester derivatives **1–5** (Scheme 1). After purification by column chromatography, the major product 6,6'-diester **1** was isolated in a 39 % yield, whereas the other products were obtained in much lower yields. Esterification of the secondary hydroxyl groups at the relatively small core carbohydrate unit proceeded with difficulty, because the disaccharide moiety becomes increasingly sterically hindered as multiple esterification with the bulky dipeptide moiety proceeds, and pentaacylated trehalose **5** was obtained in very low yield (3 %). Another problem connected with the esterification of sugars is acyl migration,[23] which was particularly favored in the partially esterified trehalose products during purification on a silica gel column. An additional problem was the separation of isomeric esters, which was barely possible. The identity and degree of acylation in esters **1**-**5** was confirmed by NMR and MS analysis. The exact dipeptide group position was confirmed by NMR only in 6,6'-diester **1** and 2,6,6'-triester **2**, whereas NMR analysis of products **3–5**

indicated mixtures of numerous trehalose dipeptide isomers. The chemical shift data in DMSO-d_6 solution for esters **1** and **2** are summarized in Tables I and II. It can be presumed that asymmetrically substituted trehalose esters, especially those with a low degree of substitution (**3** and **4**), readily undergo acyl migration, a process frequently observed during the synthesis and purification of sugar esters.[24]

It can be expected that in addition to the differences in reactivity of individual sugar hydroxyls, characteristics of the acylating reagent also contribute to the distribution of trehalose esters. To explore the influence of peptidyl moiety bulkiness on the number and position of trehalose hydroxyl group substitution, we decided to use a sterically less demanding dipeptide. Thus, the pentachlorophenyl ester of *N-tert*-butyloxycarbonyl-glycyl-glycine was reacted with α,α-trehalose in the presence of imidazole as a catalyst, using the same conditions as described for Boc-Phe-Met-OH (Scheme 1). Compounds **6–10** were isolated from the reaction mixture and characterized on the basis of NMR and MS studies. Comparison of Boc-Gly-Gly-OH and Boc-Phe-Met-OH derived trehalose esters (Scheme 1) points to significant differences in both the number and position of disaccharide O-substitution. As expected, the compound with the lowest degree of substitution is the one with the dipeptide moiety occupying the two primary hydroxyl groups of the disaccharide molecule (compound **6**, Scheme 1). In contrast to Boc-Phe-Met-OH-derived trehalose esters, the next higher substituted Boc-Gly-Gly-OH derived trehalose ester was identified as tetrasubstituted ester **7**. Further, the next two trehalose esters were, based on NMR and MS data, identified as pentasubstituted trehalose esters **8** and **9**. NMR data (further in text) point to 2,2',6,6' substitution in both esters. The fifth site in compound **8** was confirmed to be the hydroxyl group at C3. In contrast to ester **8**, the exact position of the fifth substitution site in ester **9** could not be undoubtedly established by NMR and MS data, but it can be presumed that compound **9** is the 2,2',4,6,6'-pentaester. Finally, the highest degree of substitution was found in compound **10**, identified as 2,2',4,4',6,6'-hexaester. It should be underlined that the relative distributions of the esters highly depend on the work-up procedure. Yields presented in Scheme 1 were obtained after purification by column chromatography, without acidification and extraction. When aqueous work-up is applied before column chromatography, the yield of 6,6' diester **6** is dramatically reduced (47 % → 4 %) with slightly increased yields of other higher substituted esters, as a result of combined acyl migration and extensive hydrolysis.

Comparison of compounds **1–5** with **6–10** revealed distinct differences in both the number of dipeptide units attached to the trehalose core and the position of substitution. After substitution of the primary C66' hydroxyl groups, the C2 position is the second site of choice. This finding is in agreement with the higher reactivity of the C2 hydroxyl group in the esterification reaction compared with other secondary groups, as observed in methyl α-D-glucopyranoside[12] and 4,6:4',6'-di-*O*-benzylidene α,α-trehalose.[25] However, the symmetrical 2,2',6,6'-tetrasubstituted trehalose ester was obtained with Boc-Gly-Gly-OH (compound **7**), while the asymmetrically substituted 2,6,6'-triester **2** was isolated with Boc-Phe-Met-OH. Considering trehalose symmetry and the proximity of hydroxyls at C2 and C2', it is reasonable to presume that two peptides attached at the C2 and C2' positions will line up in the same direction close to each other. This arrangement was achieved with Boc-Gly-Gly-OH; however, no 2,2',6,6'-tetraester formation was observed in the Boc-Phe-Met-OH line. These results suggest that, in the latter case, acyl donor approach to the C2' position is restrained by the sterically demanding dipeptide unit already occupying the C2 position. The degree of substitution in the other isolated esters is also in agreement with the above-mentioned influence of acyl agent bulkiness. All trehalose esters obtained with Boc-Phe-Met-OH are asymmetrically substituted (with the exception of **1**). In the Boc-Gly-Gly-OH line, however, acylation of the two primary and two secondary hydroxyls still leaves sufficient space for further substitutions and ends up with the two pentasubstituted esters, **8** and **9**. Furthermore, even hexasubstituted symmetrical ester **10** was isolated, with dipeptide units occupying the 2,2',4,4',6,6' hydroxyl positions. Although a large excess of the acylating reagent was applied, octasubstituted esters were not formed with either of the dipeptides examined, demonstrating that steric congestion leads to serious synthetic problems even with small linear peptides. Since the same molar proportions of reactants, the same solvent, acyl acceptor (trehalose) and type of activated ester (pentachlorophenyl) are used with both dipeptides studied here, it is reasonable to presume that the structure of the acylation reagent directs the distribution of acylating products.

Enzymatic synthesis of sucrose and trehalose amino acid esters revealed different acylation patterns for the two disaccharides.[9] While sucrose was acylated only at one or two of the three primary hydroxyls, trehalose was acylated at both primary (6,6'-OH) and only one secondary (3-OH) position. In contrast, chemical esterification of sucrose in aqueous medium gave mono-, di-, tri- and even higher substituted esters, depending on the reaction conditions.[24] Our previous study on the esterification pathway of methyl-α-D-glucopyranoside under the same conditions applied in the present work, showed that the reactivity of the OH groups in this non-reducing sugar decreases in the order 6-OH>2-OH>3-OH.[12] In summary, it can be concluded that the product distribution found for trehalose-centered dipeptide esters is in accord with

the results on hydroxyl group reactivity established for methyl-α-D-glucopyranoside. However, lower regioselectivity, resulting in a higher degree of substitution, was observed than for the monosaccharide type esters.

The trehalose derivatives presented here can be considered templates of sugar-based modules for the study of controlled delivery of bioactive components *in vivo*. Namely, in therapeutic drug delivery, controlled drug release is of the utmost importance. Many studies have shown that, among the carbohydrates usually used for drug release, trehalose and its esters have superior stabilizing properties compared to sucrose and especially glucose.[26,27] For further elongation of the peptide moieties in the prepared trehalose derivatives, Boc removal is a necessary step. In order to gain an insight into deprotection efficiency, we have performed deprotection of 6,6'-di-*O*-(Boc-Gly-Gly-)-α,α-trehalose (**6**) in a TFA-water (9:1) mixture, and we obtained 6,6'-di-*O*-(Gly-Gly-)-α,α-trehalose in quantitative yield (data not shown). However, some precautions are necessary, since it is known that N-terminally deprotected dipeptide esters readily undergo diketopiperazine formation.[14,28,29] Thus, the deprotection step is best performed immediately before the elongation step.

NMR Analysis of Trehalose Esters 1–10

The presence of peptide groups attached to trehalose hydroxyls results in a large number of protons in a relatively narrow range of chemical shifts, difficulties being more pronounced when moving from lower to higher substituted trehalose derivatives.

Analysis of the ^{13}C NMR spectra of compounds **1** and **6** and comparison with the spectrum of unsubstituted trehalose revealed a downfield shift for the C66' carbons (Δ 2.8 ppm, Table I) and an upfield shift for the C55' carbons (Δ 3.7 ppm). In addition, the H66' protons were also shifted to lower field (Δ 0.7 ppm) accompanied by a smaller shift in the same direction (Δ 0.3 ppm) for the neighboring H55' protons (Table II). Integration of the dipeptide amide protons and comparison with suitable well resolved trehalose protons gave a peptide:trehalose ratio of 2:1. Taken together, these data allowed identification of compounds **1** and **6** as 6,6'-diesters.

The NMR spectrum of trehalose ester **2** showed two signals for the anomeric carbon atoms at 91.57 and 94.77 ppm (Table I) and downfield shifts for C2 as well as for C66', indicating 2,6,6'-esterification of the trehalose molecule. The large downfield shift for H2 (Δ 1.4 ppm), while the chemical shift of H2' was unchanged, confirmed the asymmetrical esterification with Boc-Phe-Met-OH in compound **2**.

The ^{1}H and ^{13}C NMR spectra of compounds **3–5** were extremely complex, demonstrating that in trehalose partially acylated with bulky Boc-Phe-Met-OH moieties, a massive acyl migration occurred, giving rise to a mixture of different isomers. The dipeptide to trehalose ratio was determined for each compound from the ^{1}H NMR spectrum by comparing the amide protons with the well resolved protons of the trehalose moiety. The ^{13}C and ^{1}H NMR chemical shifts of esters **3–5** are given in the Experimental section.

Trehalose ester **7** was assigned based on COSY and HMQC experiments. Close inspection of the anomeric region showed shifts of the C11' signals to higher field (90.57 and 91.92 ppm, Table I) relative to both unsubstituted trehalose and diester **6**, and at the same time shifts

TABLE I. ^{13}C NMR chemical shifts for the trehalose moiety in Boc-Phe-Met-OH derived esters (**1**, **2**) and Boc-Gly-Gly-OH derived esters (**6**, **7**, **8** and **10**)[(a)]

Carbon atom	Trehalose	6,6'-diester	2,6,6'-triester	6,6'-diester	2,2',6,6'-tetraester	2,2',3,6,6'-pentaester	2,2',4,4',6,6'-hexaester
		1	**2**	**6**	**7**	**8**	**10**
C1	93.53	93.68	**91.57**	93.43	**90.57**	91.23	91.22
C1'	93.53	93.68	94.77	93.43	**91.92**	91.43	90.58
C2	72.03	71.31	**73.74**	71.66	**73.52**	70.28	**73.18**
C2'	72.03	71.31	71.54	71.66	**73.52**	72.75	**73.18**
C3	72.90	72.62	**69.92**	72.46	70.27	**72.67**	69.86
C3'	72.90	72.62	73.16	72.46	70.30	69.76	70.28
C4	70.52	70.04	70.58	70.16	69.78	**67.11**	**72.73**
C4'	70.52	70.04	70.58	70.16	69.78	69.83	**72.73**
C5	73.31	**69.61**	**70.22**[(b)]	**69.61**	69.88	69.94	69.77
C5'	73.31	**69.61**	**70.40**[(b)]	**69.61**	69.88	69.94	69.77
C6	61.17	**64.02**	**64.11**	63.95	**63.23**[(b)]	**62.78**[(b)]	**63.40**[(b)]
C6'	61.17	**64.02**	**64.11**	63.95	**63.73**[(b)]	**63.41**[(b)]	**63.84**[(b)]

[(a)] In DMSO-d_6 at room temperature.
[(b)] Could be reversed.

TABLE II. ¹H NMR chemical shifts for the trehalose moiety in Boc-Phe-Met-OH derived esters (**1**, **2**) and Boc-Gly-Gly-OH derived esters (**6**, **7**, **8** and **10**)[a]

Proton	Trehalose	6,6'-diester	2,6,6'-triester	6,6'-diester	2,2',6,6'-tetraester	2,2',3,6,6'-pentaester	2,2',4,4'.6,6'-hexaester
		1	**2**	**6**	**7**	**8**	**10**
H1	4.64	4.83	4.99	4.85	4.97	5.09	4.97
H1'	4.64	4.83	4.86	4.85	4.97	5.01	4.99
H2	3.23	3.27	**4.59**	3.22	**4.57**	**4.82**	**4.59**
H2'	3.23	3.27	3.32	3.22	**4.57**	**4.60**	**4.59**
H3	3.55	3.54	3.76	3.57	3.75	**5.25**	3.80
H3'	3.55	3.54	3.52	3.57	3.70	3.73	3.70
H4	3.13	3.13	3.20	3.11	3.26	3.57	**5.34**
H4'	3.13	3.13	3.20	3.11	3.26	3.27	**5.31**
H5	3.64	3.95	3.70	3.95	4.03	4.06	4.82
H5'	3.64	3.95	3.70	3.95	4.03	_[b]	4.84
H6	3.47/3.55	**4.16**	**4.12/4.23**	**4.05/4.25**	**4.11/4.30**	**4.16/4.30**[c]	**4.14/4.28**
H6'	3.47/3.55	**4.16**	**4.12/4.23**	**4.05/4.25**	**4.15/4.22**	**4.20/4.28**[c]	**4.14/4.28**

[a] In DMSO-d_6 at room temperature.
[b] Not assigned.
[c] Could be reversed.

of the C22' signals to lower field (73.52 ppm). Chemical shifts of C55' and C66' are within the same region as found for diester **6**. The most important finding in the ¹H NMR spectrum of ester **7** is a large downfield shift for the H22' protons (4.57 ppm) along with a small shift for the neighboring protons (H11' and H33') in the same direction (Table II). These data, together with the peptide:trehalose 4:1 ratio (based on integration) suggest that compound **7** is the 2,2',6,6'-tetrasubstituted trehalose ester.

Even at first sight, the ¹³C and ¹H NMR spectra of trehalose ester **8** differ from those of esters **6** and **7** by the presence of two sets of resonances, particularly notable in the proton 1D and 2D spectra. Figure 1A shows part of the COSY spectrum with designated key correlations. The lower field anomeric proton (5.09 ppm, Table II) shows a cross peak with the lower field H2 proton at 4.82 ppm, which is in correlation with the highly downfield-shifted H3 at 5.25 ppm (dashed lines at Figure 1A). Accordingly, the upper field anomeric proton at 5.01 ppm correlates with the upper field H2' proton (4.60 ppm), which shows correlation with H3' at 3.73 ppm (dotted lines in Figure 1A). Chemical shifts of the H22' protons are in agreement with O-substitution at positions 2 and 2', while the large downfield shift of H3 and the unchanged chemical shift of H3' point to esterification at the 3-position. Integration of the proton spectrum gave a peptide:trehalose ratio of 5:1, which is in agreement with 2,2',3,6,6'-pentaester structure **8**. Influence of asymmetric substitution on the carbon chemical shifts of ester **8** can be easily followed by comparison with those of the symmetrically substituted ester **7** (Table I). Substitution at the 3-position caused a downfield shift for C3 (Δ 2.4

ppm) and accordingly Δ 3.24 ppm and Δ 2.67 ppm upfield shifts for the adjacent C2 and C4.

Lack of sugar cross correlations in the 2D spectra of trehalose ester **9** made it impossible to assign undoubtedly all proton and carbon resonances. However, comparison with the NMR data of other trehalose esters allows us to speculate about its structure with a certain degree of confidence. The anomeric region in the ¹H spectrum as well as the H22' protons show two distinct sets of resonances, as seen in the spectra of ester **8**. Integration of suitable, well resolved peaks gave a 5:1 peptide:trehalose ratio. Chemical shifts of H2 and H2' at 4.86 and 4.63 ppm (see Experimental section) are in agreement with substitution at positions 2 and 2'. Since the fifth site of substitution in pentaester **8** is assigned to C3, it is reasonable to deduce that compound **9** is a 2,2',4,6,6'-pentaester.

A single set of resonances in the spectra of trehalose ester **10** points to symmetrical distribution of dipeptide units at the two trehalose rings (Figure 1B). The H22' protons are found at 4.59 ppm and correlate with the H33' protons at 3.80 and 3.70 ppm. Their correlation with H44' at 5.34 and 5.31 ppm indicates that positions 4 and 4' are occupied by peptide moieties. Moreover, the H55' protons, placed in-between two substituted sites, are also shifted downfield and found at 4.82 and 4.84 ppm. The peptide:trehalose ratio of 6:1 is in agreement with the identification of **10** as a 2,2',4,4',6,6'-hexaester. In the ¹³C NMR spectrum, all carbons at substituent-bearing positions are shifted downfield, while those nearby are found at a higher field (Table I).
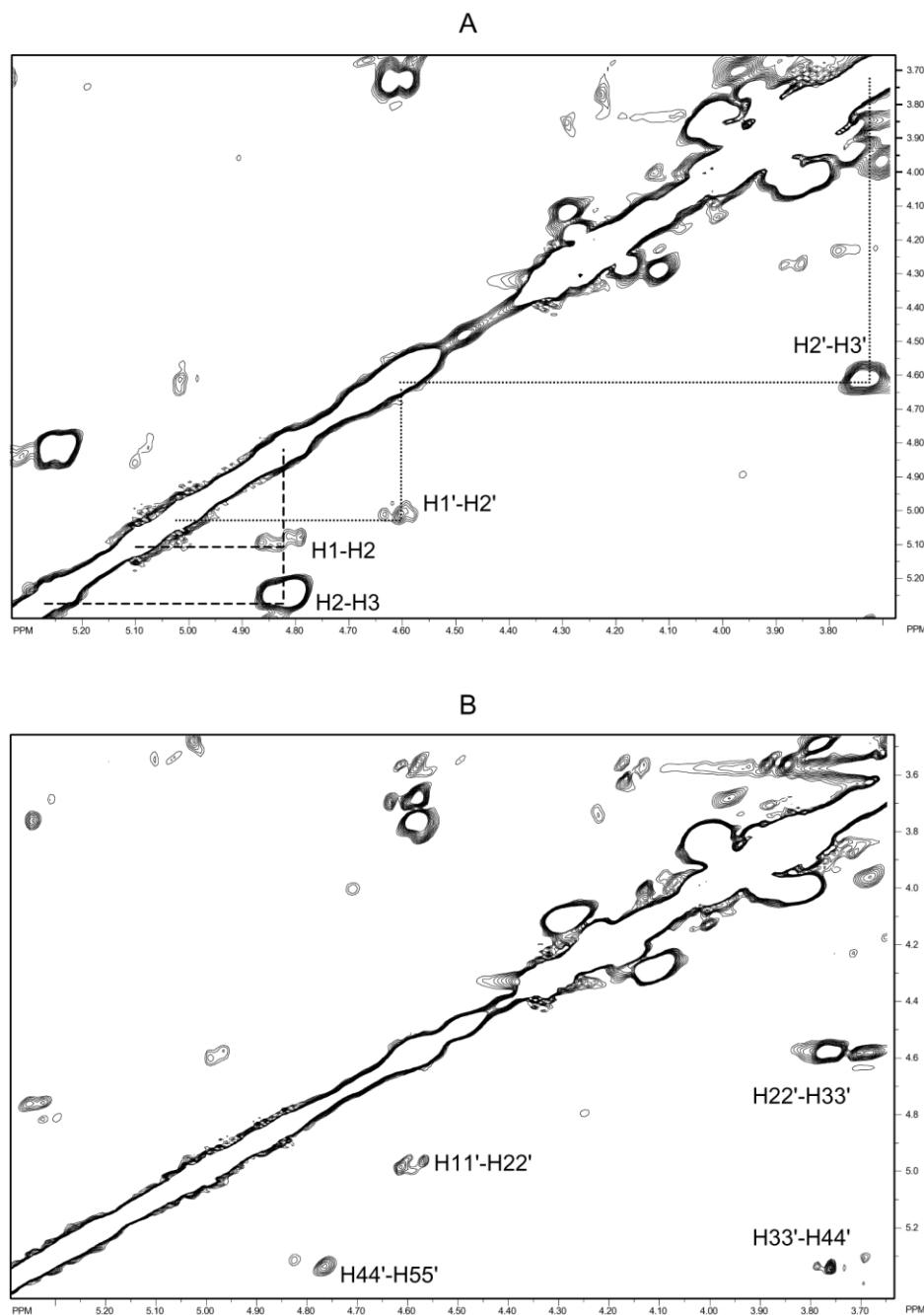
A

B

Figure 1. Part of the COSY spectra of (A) 2,2′,3,6,6′-pentaester **8** and (B) 2,2′,4,4′,6,6′-hexaester **10**.

MS Analysis of Trehalose Esters *1–10*

Full scan mass spectra of all compounds were recorded in positive and/or negative ion modes and were supplemented with MS/MS and MS^3 spectra. In positive ion mode, all compounds show pseudomolecular ions in the form of sodium adducts, [M+Na]$^+$, in agreement with the calculated values. The mass spectra of trehalose esters **2** and **3** in positive ion mode show identical values for the pseudomolecular [M+Na]$^+$ ions at *m/z* 1499, thus confirming the structure with 3 dipeptide units attached to

the trehalose core. Since the structures of Boc-Gly-Gly-OH derived trehalose esters were determined undoubtedly with respect to the number and position of substituted sites, their fragmentation behavior was studied in more detail in order to explore the utility of mass spectrometry for the characterization of trehalose-related isomeric esters. In the case of trehalose ester **10**, the pseudomolecular ions obtained in positive and negative ion modes are unstable and appear in very low intensities, thereby preventing further tandem mass spectrometric studies.
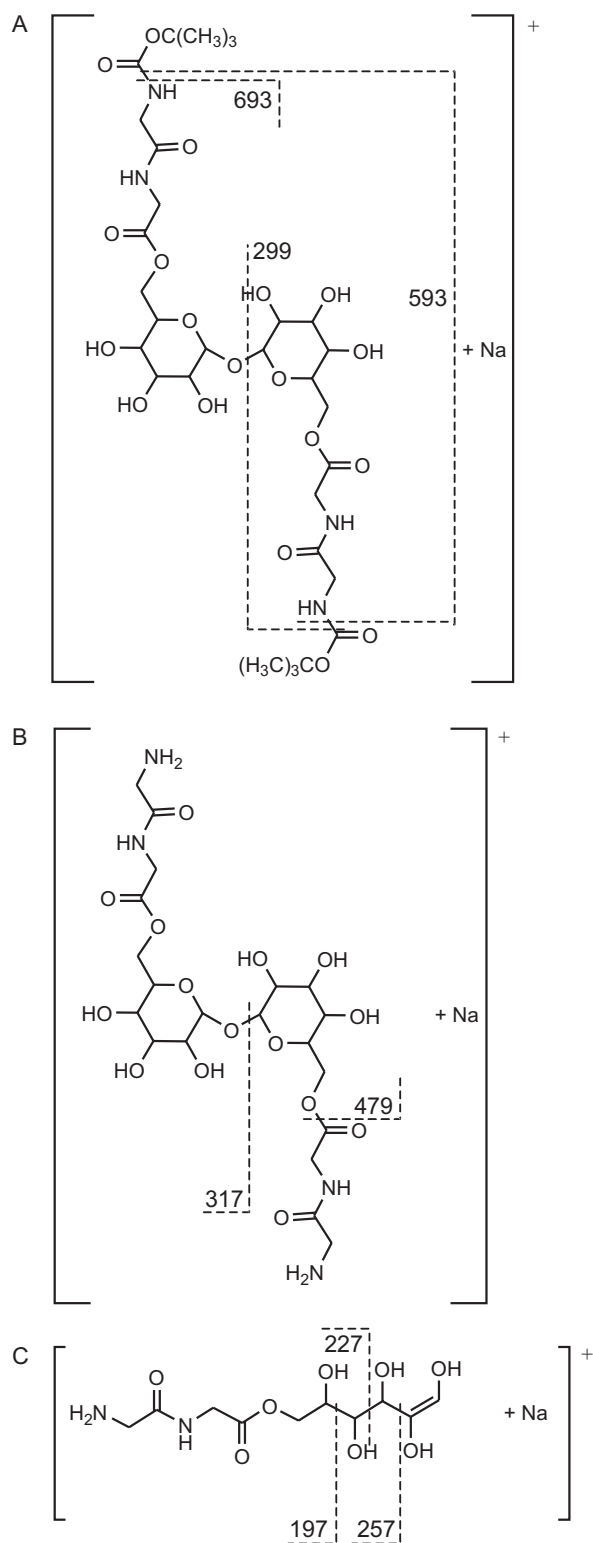
Figure 2. (A) Fragmentation of the pseudomolecular [M+Na]+ ion m/z 793 of diester **6**; (B) Fragmentation of the m/z 593 ion in the MS³ of diester **6**; (C) Structure and fragmentation of the m/z 317 ion in the MS³ of diester **6**.

The MS/MS spectrum of the pseudomolecular [M+Na]+ ion (m/z 793) of diester **6** shows two consecutive losses of 100 Da fragments originating from the cleavage of the $(CH_3)_3COCO$ moiety from the N-terminal part of the peptide (Figure 2A). The appearance of the ion at m/z 299 can be explained by the cleavage of the glycoside bond together with the 100 Da loss. Attempting to gain a closer insight into the fragmentation of **6**, we provide a collision-induced dissociation spectrum of the product ion at m/z 593, i.e., the MS³ spectrum of **6**. Inspection of this spectrum suggests two fragmentation pathways. Loss of a dipeptide unit (114 Da) gives rise to ion m/z 479 (Figure 2B), which undergoes loss of a water molecule affording m/z 461, and further CHOH loss (30 Da) to yield m/z 431. The most abundant ion in the spectrum is m/z 317, resulting from cleavage of the glycoside bond. This fragment acts as a precursor of further consecutive losses of HOCH=CHOH and two CHOH units giving ions m/z 257, 227 and 197, respectively (Figure 2C). All of these data are consistent with the structure of 6,6'-diester **6**.

Mass spectra in negative ion mode were taken at different collision energies (see Experimental section), but generally the only difference can be seen in the intensities of certain fragment ions. The tandem mass spectrum of the molecular [M–H]⁻ ion of diester **6** at m/z 769 shows the most abundant ions at m/z 695 and 621, arising from the loss of a $(CH_3)_3CO$ unit (74 Da) from the Boc-peptide N-terminus (Figure 3). Consecutive losses of two peptide chains (214 Da) give rise to low intensity ions at m/z 555 and 341. Cleavage of a single peptide moiety from the m/z 695 generates an ion at m/z 481, which further undergoes losses of one and three water molecules, to yield m/z 463 and 427, respectively. Also, m/z 481 acts as a precursor of ions m/z 361 (120 Da, four CHOH units) and m/z 301 resulting from the cleavage of the glycoside bond.

The MS of tetraester **7** in positive ion mode shows the pseudomolecular [M+Na]+ ion at m/z 1221, thus confirming the structure with 4 peptide units attached to the trehalose core. MS/MS of the [M+Na]+ ion m/z 1221 shows only ions at m/z 1121, 1021, 921 and 821, arising from consecutive losses of peptide chains, without any additional structural information. In negative ion mode, MS/MS of the molecular [M–H]⁻ ion at m/z 1197 affords a complex network of fragment ions arising from combined losses of peptide moieties (214 Da), $(CH_3)_3CO$ units (74 Da), and water molecules. Unfortunately, tetraester **7** turned out to be quite unstable under the collision conditions applied, which prevented a more detailed study of its fragmentation pattern.

Pentasubstituted trehalose esters **8** and **9** have identical molecular masses and accordingly give pseudomolecular [M+Na]+ ions at m/z 1435. Since MS/MS of m/z 1435 show only losses of $(CH_3)_3COCO$ units (100 Da), as seen with the previously mentioned esters, we attempted to get additional structural information by looking at the fragmentation behavior of m/z 935, an ion obtained
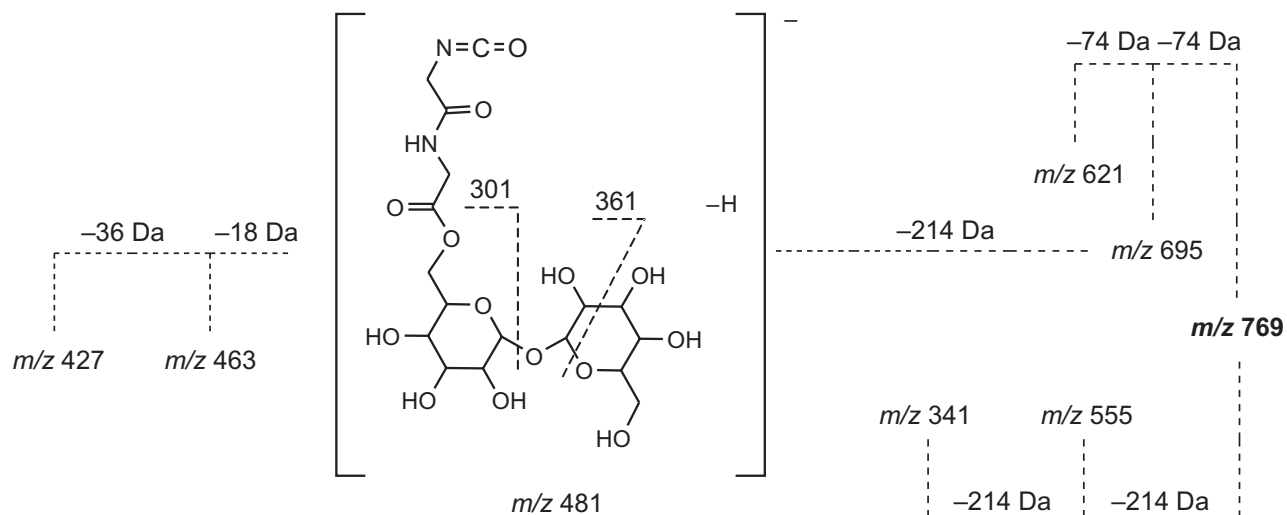
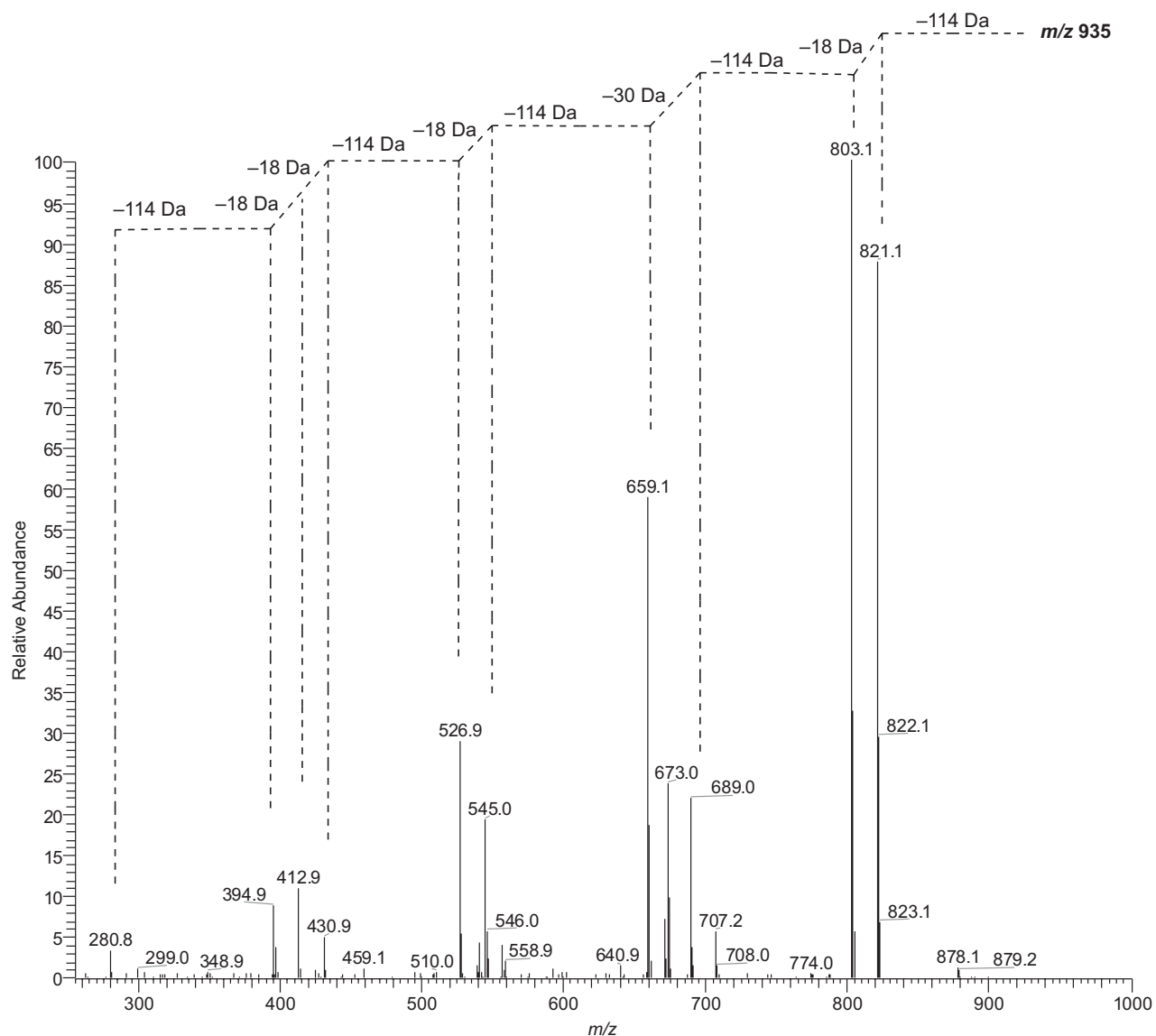Figure 3. Fragmentation of the molecular [M–H]⁻ ion at *m/z* 769 of diester **6**.

Figure 4. Positive ion MS³ of the *m/z* 935 of pentaesters **8** and **9**.

after cleavage of all five (CH$_3$)$_3$COCO moieties. MS^3 of the m/z 935 ions derived from trehalose ester **8** and **9** show identical fragmentation patterns (Figure 4). In addition to the low intensity ions m/z 878 and 746 corresponding to the loss of glycine moieties (57 Da) from m/z 935 and 803, respectively, the remaining fragments arise from the cleavage of the peptide (114 Da), CHOH moiety (30 Da) and water (18 Da). In negative ion mode, MS/MS of the molecular [M–H]$^-$ ion at m/z 1411 of both esters **8** and **9** revealed rich fragmentation patterns as observed with ester **7**, but without characteristic structural features.

CONCLUSIONS

The results presented here show that the biologically relevant α,α-trehalose can be subjected to successive chemical esterification reactions, acting as a multivalent core acceptor. Reactions performed under identical conditions (solvent, molar proportions of reactants, condensation method) with two, in the sterical sense, differently demanding dipeptides, Boc-Phe-Met-OH and Boc-Gly-Gly-OH, revealed the importance of the structure of the acylating reagent for the pattern of trehalose esters. In both lines, the 6,6'-diester was isolated in the highest yield. However, with Boc-Phe-Met-OH, only asymmetrical higher substituted esters were obtained; two triesters, a tetra- and a pentaester. In contrast, in the Boc-Gly-Gly-OH line, two symmetrical and two asymmetrical esters were isolated. In all higher substituted esters within this line, the C2, C2', C6 and C6' positions were occupied by dipeptide moieties. Both 2,2',3,6,6'- and 2,2',4,6,6'-pentaesters were isolated, in addition to the 2,2',4,4',6,6'-hexaester. These data imply that, under the reaction conditions applied, less bulky dipeptides are better arranged around the trehalose core, allowing a higher degree of substitution. Also, extensive acyl migration, accompanied by hydrolysis during aqueous work-up and chromatographic separation, is to be taken into account when performing esterification reactions under the described conditions.

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SAŽETAK

Sinteza dipeptidnih estera trehaloze

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Kemijska esterifikacija α,α-trehaloze s dva različita dipeptidna acilna donora, Boc-Phe-Met-OH i Boc-Gly-Gly-OH, dala je niz produkata koji se razlikuju u broju i položaju supstitucije. Oba korištena dipeptida daju 6,6'-diestere kao glavne produkte. U slučaju Boc-Phe-Met-OH dipeptida svi su viši supstituirani esteri nađeni s nesimetričnom raspodjelom dipeptidnih jedinica na dva trehalozna prstena. Nasuprot tomu, prvi viši supstituirani ester Boc-Gly-Gly-OH simetrični je 2,2',6,6'-tetraester. Ta četiri položaja ostaju supstituirana u svim ostalim izoliranim produktima, dvama nesimetričnim pentaesterima i jednom simetričnom heksaesteru. Prikazani rezultati pokazuju da su osim reaktivnosti šećernih hidroksilnih skupina i karakteristike acilirajućeg reagensa, prvenstveno steričke, od izuzetnog značaja kod predviđanja raspodjele acilnih skupina oko nereducirajuće središnje disaharidne molekule.