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Enzymatic Synthesis of β-Galactosyldipeptides and of β-1,3- Digalactosylserine derivatives using β-galactosidase

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Abstract : The transgalactosidation from lactose to dipeptides has been achieved using β-galactosidase from E. Coli as catalyst. Two series of dipeptides have been studied, each of them containing a serine residue. The best condensations occur when serine is at the N-terminal end of the dipeptide. Mild hydrolysis of the ester group of the labile glycosyl-dipeptide derivatives has been achieved using subtilisin. We also describe the condensation of lactose with β-galactosyl serine to give β-1,3-digalactosyl-serine derivatives.

There is actually growing interest in new methods for the stereospecific synthesis of glycosidic linkages between a sugar and an amino acid (or peptide) and for methods which would avoid the selective protections / deprotections necessary in the classical chemical synthesis of carbohydrates.

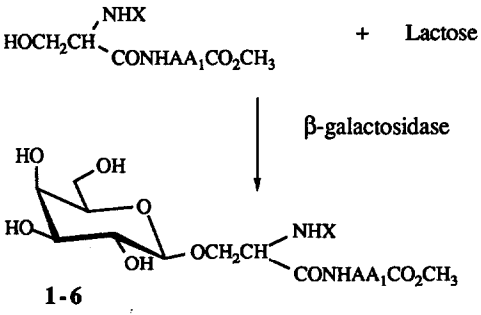
Many enzymes are now available commercially and their routine use in synthesis is becoming accepted[1]. Glycosidases have been known for a long time to transfer the glycosyl moiety of a substrate to acceptors other than water and although they are less specific than glycosyltransferases they have been used for the synthesis of several glycosides[2,3]. The β-galactosidase from *E. Coli* has been the most studied of these enzymes. It has been shown to catalyze transgalactosidation with monosaccharides, oligosaccharides, alkanols[4-6] and phenols[7]. Alkylgalactosides or lactose can be used as glycosyl donors with various primary alcohols as acceptors for the synthesis of di- or tri-saccharides[8-11]. The great advantage of these enzymatic condensations is that they are highly stereospecific. Furthermore they are performed under mild conditions, minimum protection is required and they are usually one-step preparations.

In previous papers[12] we showed that the stereospecific formation of a glycosidic bond between galactose (or glucose) and serine was possible with galactosidases (or glucosidases) as long as both the amino and the carboxyl groups of serine were protected. These results were confirmed recently by two different groups[13,14] and Sauerbrei and Thiem themselves[14] succeeded in the condensation of the unprotected serine.

The glycosyl-serine linkage being labile in basic medium we also showed[15] that clean removal of the ester group of serine could be achieved enzymatically using proteases under mild conditions. The same kind of approach has been realized recently by Kunz[16] who introduced long chain alkylesters in

the C-terminal part of peptides in glycopeptides in order to remove the protective ester group by lipases.

In an attempt to further extend the scope of these condensations we present here some of the results we have obtained in the β-galactosidase catalyzed condensations of lactose with N-protected dipeptide esters as acceptors. We also show that if 1-O-β-D-(N-allyloxycarbonyl-serine methylester)-galactopyranoside is used as acceptor the synthesis of digalactosyl-serine derivatives is possible. Two series of dipeptides have been studied. They both contain a serine residue and a second amino acid which can be glycine, alanine or serine. The serine residue is N-terminal in the first serie (Scheme 1) and C-terminal in the second serie (Scheme 2).



1-6

Scheme 1

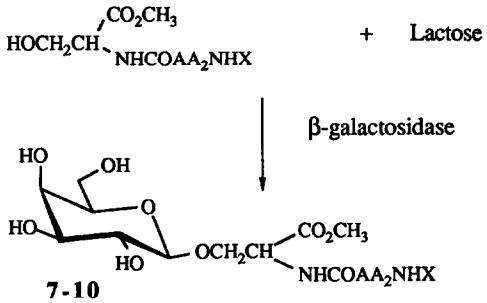
X, AA_1	Conditions[a] buffer (µl)	enzyme[b] (µl)	t/h	yield of 1-6[c] % (m.p.)
1 X=Boc AA_1=Glycine	800	90	42	13
2 X=Aloc AA_1=Glycine	800	100	40	13 (168°C)
3 X=Boc AA_1=Alanine	400	100	48	9
4 X=Aloc AA_1=Alanine	600	100	48	9 (161°C)[e]
5 X=Boc AA_1=Ser	500	100	40	11[d]
6 X=Aloc AA_1=Ser	800	100	40	11[d]

Table 1. a) amount of buffer for 0.25 mmole of lactose (see experimental part); ratio lactose / dipeptide 1/1.5; b) 0.5mg of *E. Coli* β-galactosidase (Boehringer) is dissolved in 1ml of buffer; c) after purification on silicagel; d) mixture of two regioisomers **5**a,b and **6**a,b respectively; e) **4**: $[\alpha]_D$ -29° (c 0.78, H_2O).

The allyloxycarbonyl group for N-protection is interesting in that it can be removed under mild conditions by palladium hydrostannolytic cleavage[17,18].

The condensations were performed as described before[8,12] in a sodium phosphate buffer at pH 7.8 using β-galactosidase and lactose. The peptides are more or less soluble in the buffer; consequently the reactions are runned in non homogenous medium. Surprisingly if dimethylformamide is used, much lower yields are observed. In our hands *ortho*-nitrophenyl-β-galactopyranoside as glycosyl donor gave poor results due to difficulties encountered in the purification process.

The results are summarized in Table 1 and 2. The structure of all the galactosyl-dipeptide derivatives was established by N.m.r. data and in some cases by independent synthesis (compounds **2** and **10**).



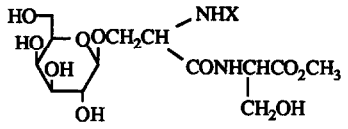
Scheme 2

	X, AA_2	Conditions[a] buffer (μl)	enzyme[b] (μl)	t/h	yield of **7-10**[c] % (m.p.)
7	X=Boc AA_2=Glycine	400	150	42	7
8	X=Aloc AA_2=Glycine	400	100	40	7 (186°C)
9	X=Boc AA_2=Alanine	800	100	3 days	5
10	X=Aloc AA_2=Alanine	800	100	3 days	5

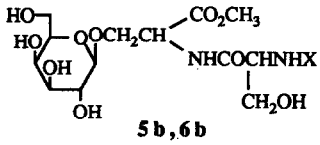
Table 2. a, b, c (same as in table 1)

It appears that the β-galactosidase catalyzed transglycosylation occurs with the two series of dipeptides. However better yields are observed if the serine residue is in the N-terminal position and the transgalactosidations are similar, in this case, to the ones obtained with serine itself. The influence of the N-protective group (t-butyloxycarbonyl or allyloxycarbonyl) is negligible as compared to the position of the serine residue.

If the peptide contains two serine residues the yields indicated are for a mixture of two products as clearly seen by N.m.r.. In fact the two primary alcohol groups of serine have reacted to give the two regioisomers **5a,b** and **6a,b** respectively in a ratio 55/45. The two isomers **5a** and **5b** have been separated by HPLC.



5a,6a



5b,6b

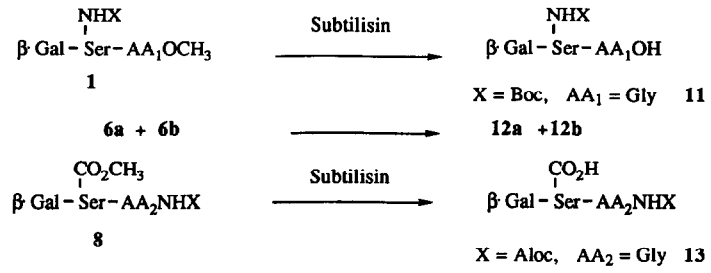
In all these cases only the β-linked dipeptides are observed.

In the second serie of dipeptides where serine is in the C-terminal position, condensations occur but in lower yields (Table 2) whether the second residue is glycine or alanine. This has already been observed in the chemical glycosylation of threonine containing peptides with galactosyl[19] or 2-azido-2-deoxy-galactopyranosyl derivatives[20]. For example, in the condensations of N-(benzyloxycarbonyl)threonyl-glycine benzyl ester and N-(benzyloxycarbonyl)-glycyl-threonine benzyl ester with 2,3,4,6-tetra-O-benzyl-D-galactopyranose using the trifluoromethanesulfonic anhydride method, a much higher yield was found for the peptide in which the glycine was on the carbonyl side of the threonine residue.

This method of stereospecific galactosidation of dipeptides is very simple; it is a one-step procedure using unexpensive lactose and the products are easily purified by column chromatography on silicagel. It could be an alternative to the classical chemical methods when authentic samples are quickly required for identification.

The glycosyl-serine linkage being labile in basic medium, the deprotection of the ester group was undertaken under mild conditions using proteases as described previously for β-galactosyl-serine methyl ester[12]. Subtilisin was chosen since papain gave cleavage of the peptide bond before hydrolysis of the ester occurred. Furthermore it is known that subtilisin displays a high ratio of esterase to protease activity and methyl esters of some N-protected di or tripeptides have been removed using this enzyme[21].

In fact, complete ester hydrolysis of the galactosyl-dipeptide derivatives **1** and **8** was observed using subtilisin at neutral pH.

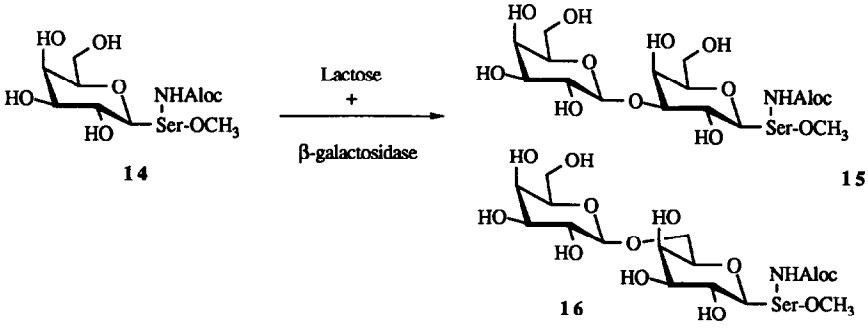


The expected galactosyl-dipeptide acids were obtained with very little cleavage of the peptide bond (<5%) for the derivatives of the first serie (acid **11**) and around 15 % for the second serie of dipeptides (acid **13**). Furthermore the β-galactosyl-dipeptide linkage was left intact. Hydrolysis was also performed on the mixture of galactosyl-diseryl derivatives **6a** + **6b**: clean hydrolysis was observed which gave a mixture **12a** + **12b** of the expected acids.

In order to extend the enzymatic glycosylation to the synthesis of disaccharides with serine linkages we subjected β-galactosyl serine itself to further transglycosylation reaction using lactose and β-galactosidase. The regioselectivity of glycosidase-catalyzed formation of disaccharides is known to depend strongly on the nature of the glycosyl acceptor. Nilsson[8] has shown that the preponderant formation of other than (1→6) linkages can be effected with glycosidases which normally give (1→6) linkages. Thus if *nitro*-phenyl-β-glycosides are used as glycosyl donors, the condensation of methyl-α-D-galactopyranoside with β-galactosidase gives mainly the (1→6) linked digalactoside whereas methyl-β-D-galactopyranoside gives mainly the (1→3) linked digalactoside. The regioselectivity also depends on the source of the β-galactosidase used (*E.Coli* or *A.oryzae*) as reported recently[14].

We chose lactose as glycosyl donor and the 1-O-β-D-(N-allyloxycarbonyl-L-serine methylester)-galactopyranoside **14** (prepared previously[12]) as substrate. The reactions were performed with *E.Coli* β-galactosidase in a mixture of phosphate buffer pH 7.8 and dimethylformamide (30%) as described by Nilsson[8]. The condensation takes place and a mixture of (1→3) and (1→6) digalactosyl-serine derivatives **15** and **16** are obtained in amounts of 13% for **15** and 6% for **16**. They have been purified easily by chromatography on silicagel and identified unambiguously by ^{13}C-N.m.r. spectroscopy by comparison with litterature data on different β-linked digalactosides[14,22-24]. In the absence of organic cosolvent no condensation takes place.

If *o*-nitrophenyl-β-galactopyranoside is used as glycosyl donor instead of lactose poor results are obtained due to difficulties in the separation of the desired derivatives **15** and **16** from the *o*-nitro-phenyl-digalactosides formed in a competitive reaction.



14 **15** **16**

Again the enzymatic synthesis of the (1→3) digalactosyl-serine derivative **15** is interesting and competitive with the classical methods since numerous steps would be required for the selective

deprotection of the 3-position of galactosyl-serine and the global yields for similar products is no more than 5% [25].

Experimental

β-galactosidase (E. Coli) was obtained from Boehringer. A solution of β-galactosidase was prepared by dissolving the enzyme powder (0.5mg) in 1 ml of 0.03M sodium phosphate buffer (pH 7.8) that contained mM $MgCl_2$ and 5mM dithiothreitol. The Boc-dipeptide esters were prepared by the classical method with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole[26]. The allyloxycarbonyl group was introduced on the dipeptide ester as described in the litterature for the t-butyloxycarbonyl group[27]. Lactose hydrolysis and galactosyl transfer were monitored, by t.l.c. on Silica Gel F_{254} with 2-propanol-aqueous 20%NH_3-water (7:1:2) and detection was made by charring with sulfuric acid. ^{1}H-N.m.r. spectra (300.134 MHz, 3-(trimethylsilyl)propionic acid, sodium salt as standard) were recorded on a Bruker instrument. Chemical shifts for ^{13}C-N.m.r. (75.47 MHz) are given relative to Me_4Si with 1,4-dioxane as spectral reference (δ *vs* Me_4Si, 67.86 p.p.m.). Cosy spectra (proton-proton shift correlations) as well as DEPT experiments were performed for the assignments of the peaks.

1-O-β-D-(N-t-butyloxycarbonylseryl-glycine methylester)-galactopyranoside 1
In a typical experiment 170mg of lactose (0.5mmole) were incubated at 37°C, with 210mg of Boc-Ser-Gly-OMe (0.75mmole) in 1.6ml of buffer (sodium phosphate buffer pH 7.8 containing mM $MgCl_2$ and 5mM dithiothreitol) and 200µl of β-galactosidase (10 units). After 42 hours the mixture was adsorbed on silicagel and subjected to column chromatography on silicagel (Merck: 0.040-0.063 mm). Elution was realized by using a gradient of CH_2Cl_2-methanol (0-5%), to remove and recover the dipeptide ester, then a slow gradient of CH_2Cl_2 with a mixture of CH_2Cl_2-methanol-ethanol-water (60: 35: 10: 8). Evaporation of the solvant gives 29mg (13%) of **1** as a semi-cristalline product, the characteristics of which are given in table 3 and table 4. $[\alpha]_D$ -3° (c 1.07, H_2O); F.a.b. m. s. $(M+H^+)$ 439.

2: $[\alpha]_D$ 0° (c 1.5, H_2O); *Anal.* calc. for $C_{16}H_{26}O_{11}N_2$: C, 45.49; H, 6.20; N, 6.63. Found : C, 45.37; H, 6.01; N, 6.58.

10: $[\alpha]_D$ -10° (c 1.35, H_2O).; *Anal.* calc. for $C_{17}H_{28}O_{11}N_2$, 1.5 H_2O : C, 44.05; H, 6.55; N, 6.14. Found : C, 44.03; H, 6.21; N, 6.26.

Note : all the galactosyl derivatives linked to N-Boc-dipeptide esters are semi-cristalline hygroscopic products (**1, 3, 7, 9**). The diseryl derivatives **5** and **6** are also oily products.

The galactosyl-diseryl derivatives **5a** and **5b** have been separated by HPLC using a Perkin-Elmer pump system with a light diffusion monitor (Touzart and Matignon, France). A column of nucleosil C_{18} 5µ was used (150 X 4.6 mm) and the products were eluted with an acetonitrile-water gradient (5-15%) ; **5b** : ^{13}C N.m.r. : the data are the same as the ones given in Table 3 for **5a**, except for the

CHα (Ser) which is at 55.57 ppm.. The mass spectrum of the mixture (chemical ionisation) is correct : $(M+H^+)$ 469.

1-O-β-D-(N-t-butyloxycarbonylseryl-glycine)-galactopyranoside 11

Product **1** (32mg, 0.073mmole) in 1ml H_2O is incubated with 7mg Subtilisin Carlsberg (Sigma) dissolved in 0.7ml H_2O at pH 7. The pH is maintained constant by addition of 0.05M NaOH with a pH stat. The end of the reaction is followed by t.l.c.. The mixture is treated by a Dowex 50W-X4 resin until pH 2 is reached and purified on a silicagel column with the same solvent as above. 21mg of product **11** are obtained (68%) : m.p. 200°C (dec.); $[\alpha]_D$ -5° (c 0.49, H_2O); ^{13}C N.m.r. data (D_2O): 176.99 (CO_2H), 172.54 (CONH), 158.28 (OCONH), 103.68 (C-1), 82.67 ($C(CH_3)_3$), 75.94 (C-5), 73.34 (C-3), 71.41 (C-2), 69.37 (C-4), 69.78 (CH_2 ser), 61.72 (C-6), 55.47 (CHα ser), 44.18 (CH_2 gly), 28.32 ($C(CH_3)_3$); F.a.b. m.s. $(M+H^+)$ 425.

The same procedure is followed for the hydrolysis of the β-galactosyl-peptide **8**. Complete hydrolysis is observed and the acid **13** is obtained in a 65% yield after purification on silicagel. The hydrolysis of **6** has been performed on a mixture of **6a** and **6b** (ratio 55/45) and gives a mixture of the two acids **12a** and **12b** in a 63% yield. They could not be separated by HPLC.

12a + 12b: ^{13}C N.m.r. data: δ, 176.45-176.35 (CO_2H), 172.37-171.99 (CONH), 158.55 (OCONH), 133.09 (CH=), 118.13 (CH_2=), 103.77-103.48 (C-1),75.80 (C-5), 73.15 (C-3), 71.32-71.26 (C-2), 69.26 (C-4), 70.59-69.52 (CH_2 bond ser), 66.89 (CH_2 allyl), 62.62-62.13-61.64-61.56 (C-6 + CH_2 free ser), 57.78-57.44 (CHα ser), 55.89-55.60 (CHα ser). F.a.b. m.s. $(M+H^+)$ of the mixture **12a + 12b** : 439

13: $[\alpha]_D$ 0° (c 1.2, H_2O); ^{13}C N.m.r. δ, 174.68 (CO_2H), 173.09 (CONH), 158.70 (OCONH), 133.28 (CH=), 118.38 (CH_2=), 103.69 (C-1), 75.98 (C-5), 73.38 (C-3), 71.47 (C-2), 69.41 (C-4), 69.71 (CH_2 ser), 67.11 (CH_2 allyl), 61.76 (C-6), 55.78 (CHα ser), 44.27 (CH_2 gly); F.a.b. m.s. $(M+H^+)$ 409.

3-O- and 6-O-β-D-galactopyranosyl-1-O-β-D-(N-Allyloxycarbonylserinemethyl ester)-galactopyranoside 15 and 16

In a typical experiment 51mg (0.15 mmole) of lactose were incubated at 37°C with 82mg (0.22 mmole) of the galactosyl-serine derivative **14** in 210 µl of a solution of β-galactosidase in the phosphate buffer at pH 7.8 (10.5 units) and 84 µl of DMF. After 36 hours two new products are observed by t.l.c.. The crude mixture was adsorbed on silicagel and subjected to chromatography on silicagel with the same solvent as above. The 1-3 digalactoside derivative **15** comes first, subsequently a mixture of **15 + 16** (the 1-6 digalactoside), then pure **16** is eluted. A second column chromatography gives **15**: 10 mg (13%) and **16**: 5 mg (6%).

15: m.p. 103°C; $[\alpha]_D$ +6° (c 1.7, H_2O); ^1H.N.m.r., δ, 5.9 (CH=), 5.2 (CH_2=), 4.55 (CH_2 allyl + H-1'), 4.5 (CHα ser), 4.4 (H-1, d, J 7.8Hz), 4.27-3.92 (CH_2 ser), 4.13, (H-4), 3.85 (H-4'), 3.77-3.85 (H-3,3'; H-5,5'; H-6,6'), 3.62 (H-2), 3.55(H-2'); ^{13}C N.m.r., δ, 173.22 (CO_2Me), 158.79 (CONH), 133.24 (CH=), 118.02 (CH_2=), 105.02-103.38 (C-1', C-1), 82.87 (C-3),

Table 3. ^{13}C N.M.R. Chemical shifts of compounds **1-5** and **7,8,10**

	Chemical Shifts (δ ppm, spectrum in D_2O)							
Carbon atom	**1**	**2**	**3**	**4**	**5**[a]	**7**	**8**	**10**
C-1	103.5	103.5	103.5	103.47	103.74	103.65	103.65	103.71
C-2	71.30	71.31	71.38	71.33	71.30	71.22	71.22	71.30
C-3	73.21	73.23	73.28	73.22	73.23	73.17	73.16	73.22
C-4	69.23	69.24	69.31	69.25	69.21	69.14	69.13	69.19
C-5	75.85	75.85	75.95	75.88	75.84	75.77	75.77	75.81
C-6	61.63	61.62	61.72	61.66	61.54	61.49	61.48	61.52
CONH	173.69	173.37	175.61	175.35	173.45	173.17	172.92	176.63
OCONH	158.09	158.56	158.17	158.40	158.20	158.67	159.09	158.28
CO_2CH_3	172.45	172.45	173.01	172.46	172.25	172.33	172.31	172.32
CH=		133.10		133.06			133.11	133.22
CH_2=		118.18		118.02			118.15	117.97
$C(CH_3)_3$	82.50		82.63		82.44	82.24		
CH_2(Ser)	69.49	69.47	69.45	69.31	69.40-62.00	69.33	69.31	69.32
CH_2 (allyl)		66.94		66.80			66.84	66.63
CHα (Ser)	55.41	55.63	55.37	55.47	57.27-53.59	53.82	53.81	53.65
CO_2CH_3	53.46	53.44	53.78	53.52	53.91	53.45	53.47	53.84
CHα (ala)			49.49	49.44				51.32
CH_2 (gly)	41.89	41.87				43.76	43.96	
$C(CH_3)_3$	28.21		28.26		28.24	28.16		
CH_3 (ala)			16.78	16.57				17.64

a) data for one of the regioisomer purified by HPLC

Table 4. ^{1}H-N.M.R.Chemical shifts of compounds **1-5** and **7,8,10**

	Chemical Shifts (δ ppm, spectrum in D_2O) and coupling constants (Hz)							
Carbon atom	**1**	**2**	**3**	**4**	**5**[a]	**7**	**8**	**10**
H-1	4.38 (d)	4.38 (d)	4.38 (d)	4.43	4.38 (d)	4.35 (d)	4.35 (d)	4.35
H-2	3.5 (dd)	3.5 (dd)	3.52 (dd)	3.5 (dd)	3.52 (dd)	3.5 (dd)	3.5 (dd)	3.5 (dd)
H-3	3.52	3.59	3.59	3.59	3.6	3.59	3.59	3.6
H-4	3.9	3.9	3.9	3.9	3.87	3.86	3.9	3.9
H-5,6,6'	3.65-3.8	3.65-3.8	3.65-3.8	3.65-3.8	3.65-3.8	3.65-3.8	3.65-3.8	3.65-3.8
CHα (Ser)	4.36	4.42	4.3	4.43	4.2, 4.75	4.7	4.75	4.7
CHα (ala)			4.45 (q)	4.43				4.15
CH_2 (Ser)	3.88-4.15	3.95-4.18	3.88-4.12	3.88-4.15	3.92-4.34, 3.82	3.92-4.3	3.92-4.32	3.85-4.32
CH_2 (Gly)	4.02	4.02				3.88	3.88	
CH=		5.95 (m)		5.95			5.95	5.95
CH_2=		5.3 (m)		5.3			5.3	5.3
CH_2 (allyl)		4.6 (m)		4.6			4.6	4.6
CH_3 (ala)			1.4	1.42 (d)				1.4 (d)
$J_{1,2}$	7.6	7.75	7.74	7.74	7.75	7.75	7.75	7.75
$J_{2,3}$	9.83	9.84	9.86	9.84	9.84	9.85	9.84	9.84
$J_{3,4}$	3.3	3.3	3.3	3.3		3.3	3.3	3.3
JCH_3 (ala)			7.3	7.3				7.3

a) data for one of the regioisomer purified by HPLC

75.78-75.52 (C-5', C-5), 73.23 (C-3'), 71.75-70.52 (C-2', C-2), 69.63 (CH_2 ser), 69.28-69.04 (C-4', C-4), 66.89 (CH_2 allyl), 61.67-61.57 (C-6', C-6), 55.07 (CHα ser), 53.93 CO_2CH_3). F.a.b. m.s. ($M+H^+$) 528.

Anal. calc. for $C_{20}H_{33}O_{15}N$, 2.5 H_20 : C, 41.91; H, 6.55; N, 2.45. Found : C, 41.65; H, 6.27; N, 2.37.

16: 1H N.m.r., δ,5.95 (CH=), 5.3 (CH_2=), 4.53 (CH_2 allyl), 4.5 (CHα ser), 4.41-4.38 (H-1,1', 2 d, J=7.8 Hz), 4.17-3.91 (CH_2 ser) 3.62 H-3,3'), 3.48 (H-2,2'); ^{13}C N.m.r., δ, 173.17 (CO_2Me), 158.77 (CONH), 133.22 (CH=), 118.06 (CH_2=), 104.04-103.68 (C-1', C-1), 75.87-74.57 (C-5', C-5), 73.40-73.16 (C-3', C-3), 71.43-71.28 (C-2', C-2), 69.76-69.55 (C-6, CH_2 ser), 69.31 (C-4', C-4), 66.89 (CH_2 allyl), 61.76 (C-6'), 55.06 (CHα), 53.92 (CO_2CH_3). F.a.b. m.s. ($M+H^+$) 528.

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