^{13}C NMR Spectroscopy, a Useful Tool To Determine the Enantiomeric Purity of Synthetic Threonine-Containing Glycopeptides. Spectra of Diastereoisomeric α- and β-D-Galactopyranosyl-L- and -D-threonine and -L- and -D-allothreonine†

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Further studies of *O*-glycosyl amino acids by natural-abundance ^{13}C NMR spectroscopy are presented. Carbon-13 NMR spectra of diastereoisomeric α- and β-D-galactopyranosyl-L-threonine, -D-threonine, -L-allothreonine, and -D-allothreonine showed distinct anomeric (C1) and threonine methyl (C′γ) signals which provide a practical means to monitor the enantiomeric purity of synthetic *O*-glycopeptides. Chemical shifts for the above carbons were shown to be closely related to the absolute configuration of both the anomeric (C1) and the aglyconic (C′β) carbons. The presence on the same molecule of two different probes (C1 and C′γ) and the importance of chemical shift differences ($\Delta\delta$C1 up to 8.5 ppm and $\Delta\delta$C′γ up to 6 ppm) allowed the facile detection of any racemization occurring at either or both of the two asymmetric centers of the threonine molecule. In its convenience and sensitivity, ^{13}C NMR spectrometry compares favorably with other routine racemization tests.

It is well-known that ^{13}C NMR chemical shifts around a glycosidic linkage depend upon conformation.[1] This observation was confirmed in the case of glycosyl amino acids[2] and glycopeptides,[3] since chemical shifts for anomeric (C1) and threonine methyl (C′γ) carbon atoms were found to be strongly affected by conformations. In a recent paper, Tori and co-workers[4] reported that ^{13}C NMR chemical shift differences resulting from glycosidation of secondary alcohols represented an original way to determine the absolute configuration of the hydroxyl group in a chiral alcohol.

Observations presented in this report, based on ^{13}C NMR studies of diastereoisomeric α- and β-D-galactopyranosyl-L-threonine, -D-threonine, -L-allothreonine, and -D-allothreonine, confirm the usefulness of ^{13}C NMR spectroscopy in this field. Consequently, we wished to prove its utility as a simple, practical routine method to test racemization occurring during either glycosylation or peptide condensation.

Results and Discussion

Racemization during the coupling of amino acid components is an important problem in the synthesis of peptides and glycopeptides. Precision of the results of testing the degree of racemization of synthetic peptides depends on the efficiency of the method used to analyze diastereoisomeric or racemic mixtures. Polarimetry,[5,6] has proved to be a valuable technique in routine analysis, with a sensitivity limit of 1–2% racemization. Ion-exchange chromatography using an amino acid analyzer has proved to be successful since sensitivity limits of 0.01% have been attained.[7] A generally applicable method utilizes the stereoselective enzymic hydrolysis of diastereoisomeric peptides by leucine aminopeptidase.[8]

Since the pioneering work by Weinstein and Pritchard,[9] ^1H NMR spectroscopy has been extensively used as a routine test for racemization. The most convenient approach is that developed by Davies and co-workers[10,11] and based upon methyl ester chemical shifts.[12,13] In its sensitivity, it compares favorably with polarimetric methods. To our knowledge, ^{13}C NMR spectroscopy has never been used to investigate racemization in peptides. We now wish to present conclusive evidence which proves this technique to be a workable, convenient racemization test.

The syntheses of benzyl *N*-(benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-D-galactopyranosyl)-L-threoninate(α-(Bzl$_4$-α-D-Gal→Z-L-Thr-OBzl)) (**1**) and β-(Bzl$_4$-β-D-Gal→Z-L-Thr-OBzl) (**2**) as well as the corresponding unprotected analogues α-D-Gal→L-Thr (**3**) and β-D-Gal→L-Thr (**4**) were described previously.[14] Essentially the same procedure was followed to prepare α- and β-D-Gal→D-Thr (**13**, **14**), α- and β-D-Gal→L-aThr (**15**, **16**), and α- and β-D-Gal→D-aThr (**17**), **18**) (see Experimental Section).

In view of the conclusions reached by Seo and coworkers,[4] we expected the ^{13}C NMR chemical shifts of anomeric carbon C1, threonine carbon C′β, and possibly C′α and C′γ to be affected by a change of configuration occurring at the aglyconic carbon (C′β). Conversely, configurational changes of C′α should mostly affect the chemical shift of C′β and C′γ, and less likely that of the anomeric carbon (C1). We have previously shown[15,16] that carbons C′β and C′α exhibit a large chemical shift pH dependence. Differences in chemical shifts up to 3.4 and 4.9 ppm were measured for C′α and C′β in α-D-galacto-

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Table I. Partial Carbon-13 NMR Chemical Shift Data for Fully Protected α- and β-D-Galactopyranosyl-L- and -D-threonines

compounds[a]	chemical shifts[b]				absolute configuration		$[\alpha]_D^{25}$, deg ($CHCl_3$)
	C1	$\Delta\delta$ C1[c]	C′γ	$\Delta\delta$ C′γ[d]	C1	C′β	
Bzl_4-α-D-Gal→Z-D-Thr-OBzl (**6**)	94.7	−4.3 (−5.0)	15.65	−3.25	*S*	*S*	+20
Bzl_4-β-D-Gal→Z-L-Thr-OBzl (**2**)	102.2	−3.0 (−2.2)	17.5	−1.4	*R*	*R*	−5.5
Bzl_4-α-D-Gal→Z-L-Thr-OBzl (**1**)	98.7	−0.3 (−1.0)	19.1	+0.2	*S*	*R*	+29
Bzl_4-β-D-Gal→Z-D-Thr-OBzl (**7**)	103.8	−1.4 (−0.6)	19.15	+0.25	*R*	*S*	*e*

[a] Abbreviations: Bzl_4 = 2,3,4,6-tetra-*O*-benzyl; Z-D-Thr-OBzl = benzyl *N*-(benzyloxycarbonyl)-D- or -L-threoninate; Bzl_4-α-D-Gal→Z-D-Thr-OBzl = benzyl *N*-(benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-D-threoninate. [b] Chemical shifts in ppm relative to Me_4Si as internal standard in $CDCl_3$. [c] Chemical shift differences were obtained as follows: $\Delta\delta$ C1 = δ (galactosylthreonine) − δ (methyl galactopyranoside). C1 chemical shifts references were 99.0 and 105.2 ppm for methyl 2,3,4,6-tetra-*O*-benzyl-α- and β-galactopyranoside, respectively. Values given in parentheses were derived by comparison with benzyl *N*-(benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl)-α- and -β-L-serine (99.7 and 104.4 ppm ppm). Negative values correspond to a shielding. [d] $\Delta\delta$ C′γ = δ (galactopyranosyl-threonine) − δ (benzyl *N*-(benzyloxycarbonyl)-L-(or -D-)threoninate (18.9 ppm). [e] Contained small amount of compound **6**.

Table II. Partial Carbon-13 NMR Chemical Shift Data for Fully Deprotected α- and β-D-Galactopyranosyl-L- and -D-threonines

compounds	chemical shifts[a]				absolute configuration		$[\alpha]_D^{25}$, deg (H_2O)
	C1	$\Delta\delta$ C1[b]	C′γ	$\Delta\delta$ C′γ[c]	C1	C′β	
α-D-Gal→D-Thr (**13**)	95.0	−5.5 (−5.3)	15.3	−5	*S*	*S*	+46
β-D-Gal→L-Thr (**4**)	101.3	−3.6 (−2.5)	18.2	−2.1	*R*	*R*	−13
α-D-Gal→L-Thr (**3**)	101.2	+0.7 (+0.9)	19.9	−0.4	*S*	*R*	+36
β-D-Gal→D-Thr (**14**)	103.4	−1.5 (−0.4)	19.1	−1.2	*R*	*S*	*d*

[a] Chemical shifts (ppm) in water with respect to 1,4-dioxane (67.86 ppm) as internal reference. [b] $\Delta\delta$ (ppm) were obtained by comparison with methyl α- and β-galactopyranoside (100.5 and 104.9 ppm). Values in parentheses were derived by comparison with galactopyranosyl-α- and -β-L-serine (100.3 and 103.8 ppm). [c] Reference chemical shift is that measured in L- or D-threonine (20.3 ppm). [d] See Table I, footnote e.

pyranosyl-L-threonine when going from the cationic (pH ≤ 2) to the anionic (pH > 11) form of the amino acid. In contrast, the chemical shifts of C′γ and anomeric carbon (C1) were virtually unaffected (≲ 0.2 ppm). Therefore, only these latter two chemical shifts could reasonably be utilized to monitor changes of absolute configuration.

Galactopyranosyl-L- and -D-threonine Derivatives. The ^{13}C NMR chemical shifts of the α- and β-D-galactopyranosyl-L- and -D-threonines are reported in Tables I and II for fully protected and fully deprotected compounds, respectively. The chemical shifts of carbons C1 and C′γ were compared with those of methyl galactopyranosides and threonine to derive the glycosidation shift as follows: $\Delta\delta$C1 = δ (galactopyranosylthreonine) − δ (methyl galactopyranoside) for the sugar moiety and $\Delta\delta$C′γ = δ (galactopyranosylthreonine) − δ (threonine) for the aglycon moiety. $\Delta\delta$C1 was also obtained by comparison with the corresponding galactosylserine. Assignments of the resonances to anomeric and methyl carbon atoms were straightforward from previous work on similar compounds.[2,14,15]

It should be noted that the absolute configuration of the anomeric carbon (C1) in galactopyranosylthreonine derivatives is *S* and *R* for the α and β anomers, respectively, whereas Cβ is *S* in D-threonine and L-allothreonine and *R* in L-threonine and D-allothreonine.

Data reported in Tables I and II have several implications for studies of enantiomeric purity of glycopeptides by ^{13}C NMR spectroscopy. Particularly noteworthy is the existence of a significant and concomitant shielding of carbon atoms C′γ and C1 when the latter and the aglyconic carbon (C′β) have the same absolute configuration. As seen in Table I, the anomeric carbon was strongly shielded (−4.3 and −3 ppm) in compounds **6** (C1(*S*), C′β(*S*)) and **2** (C1(*R*), C′β(*R*)) as was the methyl carbon of threonine (C′γ) (−3.25 and −1.4 ppm). In contrast, much lower shielding (−0.3 and −1.4 ppm) was found for C1 in compounds **1** (C1 (*S*), C′β(*R*)) and **7** (C1(*R*), C′β(*S*)). In the latter, the methyl carbon was deshielded by 0.2 and 0.25 ppm. This trend was maintained in fully deprotected compounds as well. In compounds **13** (*S*,*S*)[17] and **4** (*R*,*R*) anomeric signals were upfield by −5.5 and −3.6 ppm, respectively, whereas differences were much lower (−1.5 ppm) or even positive (+0.7 ppm) in compounds **14** (*R*,*S*) and **3** (*S*,*R*). Similar effects were noted for methyl carbon (C′γ) which was found to be strongly shielded (−5 and −2.1 ppm) in compounds **13** (*S*,*S*) and **4** (*R*,*R*) and much less shielded (−1.2 and −0.4 ppm) in compounds **3** (*S*,*R*) and **14** (*R*,*S*).

Optical rotations reported for **1**, **2**, and **6** could never have been used to identify these compounds, as seen by comparing values given for **1** and **6**. The covalent association of a molecule of L-threonine ($[\alpha]_D$ −33.9°) with a molecule of α-D-galactopyranose should have led to an optical rotation lower than that for the same α-D-galactopyranose linked to D-threonine ($[\alpha]_D$ +33.9°). In fact, as seen from Table I, the reverse is observed. Values measured for unprotected compounds **13** and **3** were in best agreement with expectations.

Shielding effects reported above were observed both in organic solution for protected compounds and in aqueous medium for unprotected analogues. This phenomenon was noted previously for 1,1′-glycosyl glycosides[2,18] and rationalized on the basis of a gauche–gauche NMR effect[19] involving anomeric carbon. It strongly indicates that conformational properties of glycosyl glycosides and glycopeptides are largely governed by thermodynamic considerations related to the peculiar nature of the cyclic acetal unit. In this regard, the exo anomeric effect[1,2,20,21]

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Table III. Partial Carbon-13 NMR Chemical Shift Data for Fully Protected α- and β-D-Galactopyranosyl-L- and -D-allothreonines

compounds[a]	chemical shifts[b] C1	Δδ C1[c]	C′γ	Δδ C′γ[d]	absolute configuration C1	C′β	$[\alpha]_D^{25}$, deg ($CHCl_3$)
Bzl_4-β-D-Gal→Z-D-aThr-OBzl (12)	103	−2.2 (−1.4)	18	+0.2	R	R	+9.5
Bzl_4-α-D-Gal→Z-L-aThr-OBzl (9)	98.2	−0.8 (−1.5)	18.1	+0.3	S	S	+64
Bzl_4-β-D-Gal→Z-L-aThr-OBzl (10)	105	−0.2 (−0.6)	19.1	+1.3	R	S	+5
Bzl_4-α-D-Gal→Z-D-aThr-OBzl (11)	100.65	+1.65 (+1.0)	18.6	+0.8	S	R	+17

[a-c] See Table I. [d] The reference chemical shift for C′γ is that measured in benzyl N-(benzyloxycarbonyl)-D,L-allothreoninate (17.8 ppm).

Table IV. Partial Carbon-13 NMR Chemical Shift Data for Fully Deprotected α- and β-D-Galactopyranosyl-L- and -D-allothreonines

compounds	chemical shifts[a] C1	Δδ C1[b]	C′γ	Δδ C′γ[c]	absolute configuration C1	C′β	$[\alpha]_D^{25}$, deg (H_2O)
α-D-Gal→L-aThr (15)	96.8	−3.7 (−3.5)	13.2	-3.95	S	S	+39
β-D-Gal→D-aThr (18)	102.5	−2.4 (−1.3)	15.6	-1.55	R	R	+1
α-D-Gal→D-aThr (17)	99.0	−1.5 (−1.3)	16.0	-1.2	S	R	+30
β-D-Gal→L-aThr (16)	102.5	−2.4 (−1.3)	16.75	-0.4	R	S	+3

[a,b] See Table II. [c] The reference chemical shift for C′γ is that measured in D,L-allothreonine (17.15 ppm).

should represent the most significant contribution.

The above results may be summarized as follows:

(i) A significant and concomitant shielding of both anomeric and methyl carbons (C1 and C′γ) implies that both C1 and aglyconic carbon (C′β) have the same absolute configuration, namely *R,R* in β-D-Gal-L-Thr (**4**) for instance. The same phenomenum with the same sequence of variation was previously observed in 1,1′-glycopyranosyl glycopyranosides.[2,18] Particularly noteworthy was the shielding of α- and β-anomeric carbons in symmetrical 1,1′-α-D-glycopyranosyl α-D-glycopyranosides (*R,R*) and 1,1′-β-D-glycopyranosyl β-D-glycopyranosides (*S,S*) as compared to the corresponding *R,S* and *S,R* isomers. It should be emphasized that this effect is not correlated with the anomeric configuration of the glycosidic bond, provided the absolute configuration is the same, because similar upfield shifts are observed for the anomeric carbons of α-D-galactopyranosyl α-D-galactopyranoside and β-D-galactopyranosyl-L-threonine.

(ii) A shielding of less than −1.5 ppm for C1 and −1.2 ppm for C′γ or a deshielding indicates opposite absolute configurations for C1 and C′β. This rule was applied to diastereoisomeric α- and β-D-galactopyranosyl-L- and -D-allothreonines.

Galactopyranosyl-L- and -D-allothreonine Derivatives. In order to test the validity of the above rule 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose was condensed with racemic benzyl *N*-(benzyloxycarbonyl)-D,L-allothreoninate (**5**). Pure benzyl *N*-(benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α- and -β-D-galactopyranosyl)-L-allothreoninate (**9** and **10**) and benzyl *N*-(benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α- and -β-D-galactopyranosyl)-D-allothreoninate (**11** and **12**) were obtained and upon hydrogenation afforded α-D-Gal→L-aThr (**15**), β-D-Gal→L-aThr (**16**), α-D-Gal→D-aThr (**17**), and β-D-Gal→D-aThr (**18**).

^{13}C NMR data for compounds **9–12** and **15–18** are presented in Tables III and IV. The chemical shift increments for compounds **15** to **18** allow a distinction between two groups. Simultaneous shieldings of carbons C1 and C′γ were observed in compounds **15** (Δδ C1 = −3.7 ppm and Δδ C′γ = −3.95 ppm) and **18** (Δδ C1 = −2.4 ppm and Δδ C′γ = −1.55 ppm). On the other hand, in compounds **16** and **17**, Δδ C′γ were less than above, and carbon C1 showed the same shielding. As a rule, the shielding effects were lower, and even deshielding effects were observed, in protected compounds compared to those measured in unprotected analogues. On this basis we assigned structures to compounds **9** to **12** and **15** to **18**. (Tables II and III).

In order to establish unambiguously the validity of such a conclusion, it was necessary to confirm these assignments by an independent means. Hence, compounds **9**, **10**, **15**, and **16** were later prepared from pure L-allothreonine (Fluka, ref 05753). Their chemical, physical, and NMR spectral data were identical with those reported for compounds obtained from the racemate (see Experimental Section).

It should be noted that the absolute configurations of anomeric carbons are easily determined either by ^{13}C or ^1H NMR spectroscopy via the anomeric status of the glycosidic bond (δC1, J_{C1-H1}; δH1, J_{H1-H2}). Therefore, distinction between diastereoisomers is reduced to determination of the absolute configuration of the aglyconic carbon. In this regard, the existence of two probes namely, anomeric carbon (C1) and threonine methyl carbon (C′γ), instead of only one represents a significant advantage. It would have been difficult to discriminate β-D-Gal→L-aThr (**16**) from β-D-Gal→D-aThr (**18**) only on the basis of the chemical shift of the anomeric carbon (C1). This can be done from the methyl signal. It should be noted again that neither optical rotation nor ^1H NMR spectroscopy would have been helpful to make these assignments. Indeed, ^1H NMR spectra of compounds **16** and **18** were remarkably similar; the largest difference in chemical shift was that found for protons H′β (0.06 ppm). No differences in coupling constants were noted except that for $J_{H\alpha-H\beta}$, which was 3.5 Hz in **16** compared to 3.3 Hz in **18** (see Experimental Section).

Racemization. Carbon-13 NMR signals of anomeric and methyl carbons are well suited to monitor an eventual racemization of the threonine molecule. Erbing and coworkers[22] had previously noted a slight difference in chemical shift (Δδ = 0.3 ppm) for the xylose anomeric carbon in *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-xylo-

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Table V. Carbon-13 NMR Chemical Shifts of Anomeric Carbon (C1) and Threonine Methyl Carbon (C'γ) in Diastereoisomeric α- and β-D-Galactopyranosyl-L- and -D-threonine and α- and β-D-Galactopyranosyl-L- and -D-allothreonine

compounds	absolute configuration			chemical shifts	
	Cl	C'β	C'α	Cl	C'γ
α-D-Gal→D-Thr (13)	S	S	R	95.0	15.3
α-D-Gal→L-aThr (15)	S	S	S	96.8	13.2
α-D-Gal→D-aThr (17)	S	S	R	99.0	16.0
α-D-Gal→L-Thr (3)	S	R	S	101.2	19.9
β-D-Gal→L-Thr (4)	R	R	S	101.3	18.2
β-D-Gal→D-aThr (18)	R	R	R	102.5	15.6
β-D-Gal→L-aThr (16)	R	S	S	102.5	16.75
β-D-Gal→D-Thr (14)	R	S	R	103.4	19.10

pyranosyl-L-serine. This difference was ascribed to partial racemization occurring at the serine α-carbon during O-deacetylation. In our case, the situation is much more favorable since chemical shift differences of up to 8.5 and 6 ppm for anomeric and methyl carbon, respectively, were observed (see Table V). Moreover, both signals are located in well-differenciated and uncrowded regions of the spectrum, allowing accurate integration.

Data reported in Table V clearly show that racemization occurring at either or both the threonine α- or β-carbons will be easily detected. Let us consider diastereoisomeric α-galactopyranosylthreonines (3, 13, 15, and 17) as an example. In these compounds, anomeric carbons (C1) have the S configuration. Any racemization occurring at the threonine β-carbon, which represents the most probable event during the glycosylation step, will result in a mixture of α-D-Gal→L-Thr (3) and α-D-Gal→L-aThr (15). Within the limits of its sensitivity, ^{13}C NMR spectroscopy will easily discriminate the above compounds on the basis of the chemical shift differences since anomeric and methyl signals are separated by 4.4 and 6.7 ppm, respectively. Racemization during peptide coupling most likely occurs at the α-carbon bearing functions involved in the coupling reaction. In this case, we would have to discriminate L-threonine from D-allothreonine derivatives. Assignments of anomeric and methyl carbons in a mixture of α-D-Gal→L-Thr (3) and α-D-Gal→D-*allo*-Thr (17) will be straightforward ($\Delta\delta$C1 = 2.2 ppm, $\Delta\delta$C'γ = 3.9 ppm).

From analysis of known diastereoisomeric mixtures, at 21 MHz using routine conditions, we were able to estimate the sensitivity of the method to 2–3% with an average accuracy of ±0.2%.

In summary, our studies show that ^{13}C NMR spectroscopy is very well suited for the analysis of diastereoisomeric mixtures of threonine-containing glycosyl amino acids and glycopeptides.[23] The advantages are as follows: (i) two different signals are available, one from each moiety; (ii) both signals are found in two different, uncrowded regions of the spectrum, which allows a facile assignment; and (iii) chemical shift differences are considerable and allow accurate analysis.

Experimental Section

Nuclear Magnetic Resonance Measurements. Carbon-13 NMR spectra were recorded on a Bruker WP 80 (20.115 MHz) spectrometer in deuterated chloroform solutions for fully protected derivatives or deuterium oxide for fully deprotected glycosyl amino acids. Chemical shifts are expressed in part per million (ppm) with respect to Me_4Si as internal standard. 1,4-Dioxane was used as internal standard in aqueous solution, and its chemical shift was set at 67.86 ppm. In this case, samples to be examined were prepared by passing an aqueous solution of the glycopeptide through a short column of Chelex-100 (H^+) ion-exchange resin. The effluent was freeze-dried and the residue dissolved in deuterium oxide.

Proton NMR experiments were performed at 400 MHz on a Bruker WH-400 spectrometer. The compounds to be examined were exchanged twice with 99.7% deuterium oxide and dissolved in 100% D_2O. Proton chemical shifts are given in ppm from Me_4Si. Acetone (2.2 ppm) was used as internal standard.

Optical rotations were recorded on a Perkin-Elmer M 241 spectropolarimeter in a 10-mm capillary cell.

Capillary melting points were determined on a Büchi aparatus and are reported uncorrected. Elemental analyses were determined by the Service de Microanalyse du Centre de Recherche de Clin-Midy, Montpellier, France.

Thin-layer chromatography was performed on silica gel plates, sprayed with 10% sulfuric acid in ethanol, followed by heating at 100 °C. Column chromatography was usually performed on silica Merck 60 (70–230 mesh) except for "flash" chromatography, which was performed on silica Merck 60 (230–400 mesh). Systems used in column or thin-layer chromatography were as follows (v/v): A, chloroform–diethyl ether 95:5; B, hexane–ethyl acetate 80:20; C, chloroform–diethyl ether 97:3.

Compounds **1**, **2**, **3**, and **4** were described elsewhere.[14]

Benzyl *N*-(Benzyloxycarbonyl)-D-threoninate (5). The hemioxalate salt of benzyl D-threoninate (Bachem) (2.5 g, 10 mmol) and sodium bicarbonate (2.1 g) were mixed in water (15 mL) and followed by the addition over a 10-min period of benzyl chloroformate (1.0 g, 11 mmol) with stirring. After 3 h at room temperature, dioxane was evaporated in vacuo and the reaction mixture extracted with ethyl acetate (2 × 100 mL). The organic phase was successively washed with 1 N hydrochloric acid (3 × 50 mL), water (50 mL), saturated sodium bicarbonate (2 × 50 mL), and water (50 mL). The dried organic solution was concentrated in vacuo to an oil which crystallized. Recrystallization from hexane–ether afforded a pure crystalline material in 80% yield: mp 70–71 °C; $[\alpha]^{20}_D$ +15° (*c* 1.0, $CHCl_3$); ^{13}C NMR δ 171.24 (CO ester), 156.96 (CO urethane), 68.0 (Cβ-Thr), 67.35 and 67.25 (CH_2 benzyl ester and benzyloxycarbonyl), 59.70 (Cα-Thr), 19.90 (Cγ-Thr). Anal. Calcd for $C_{19}H_{21}O_5N$: C, 66.47; H, 6.14; N, 4.08. Found: C, 66.50; H, 6.20; N 4.19.

Benzyl *N*-(Benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α- and -β-D-galactopyranosyl)-D-threoninate (6 and 7). The aforementioned product (1.32 g, 4 mmol) in cold (−15 °C) acetonitrile (30 mL) was mixed with trifluoromethanesulfonic anhydride (0.25 mL, 1.5 mmol) followed by the addition in portions of 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose prepared as previously described.[23] After 30 min (TLC, solvent A), the reaction was stopped by the addition of water (50 mL), and the solution extracted with ether (3 × 50 mL). The organic phase was washed with aqueous saturated sodium bicarbonate (2 × 50 mL) and water (50 mL) and dried over sodium sulfate. Evaporation in vacuo furnished an oily residue which was flash chromatographed (solvent B) to remove the excess of amino acid derivatives. The anomeric mixture of **6** and **7** (0.75 g, 85%) was then separated on silica (solvent C) to give pure **6** (0.36 g) together with an equimolecular mixture of **6** and **7** (0.35 g).

Compound **6**: $[\alpha]^{20}_D$ +20° (*c* 0, 5 $CHCl_3$); ^{13}C NMR δ 170.65 (CO ester), 156.9 (CO urethane), 94.6 (Cl-Gal), 59.2 (Cα-Thr), 15.6 (Cγ-Thr). Anal. Calcd for $C_{53}H_{55}O_{10}N$: C, 73.52; H, 6.35; N, 1.61. Found: C, 73.35; H, 6.34; N, 1.44.

Compound **7** in a mixture with **6**: ^{13}C NMR δ 170.4 (CO ester), 156.9 (CO urethane), 103.8 (C1-Gal), 59.2 (Cα-Thr), 19.15 (Cγ-Thr).

Benzyl *N*-(Benzyloxycarbonyl)-D,L-allothreoninate (8). This compound was prepared on a 10-mmol scale by using the procedure described for compound **5** except that racemic D,L-allothreonine was used: mp 67–68 °C (from hexane–diethyl ether); ^{13}C NMR δ 170.4 (CO ester), 156.6 (CO urethane), 68.85 (Cβ-Thr), 67.35 (CH_2 benzyl ester and benzyloxycarbonyl), 59.8 (Cα-Thr), 18.9 (Cγ-Thr). Anal. Calcd for $C_{19}H_{21}O_5N$: C, 66.47; H, 6.14; N 4.08. Found: C, 66.32; H, 6.08; N 4.06.

Benzyl *N*-(Benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α- and -β-D-galactopyranosyl)-L-allothreoninate (9

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and 10) and Benzyl *N*-(Benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α- and -β-D-galactopyranosyl)-D-allothreoninate (11 and 12). Procedure A. These compounds were prepared by the procedure described for compounds **6** and **7**, and the workup was similar. After the excess of amino acid derivative was removed by flash chromatography, the diastereoisomeric mixture (0.83 g, 92%) showed three distinct spots on TLC (solvent A): R_f 0.52, 0.38, and 0.26. The above mixture was chromatographed (solvent C) on silica and afforded 5 fractions.

Fraction 1 contained pure α compound **9** (0.25 g). Fraction 2 was shown to be a mixture of fractions 1 and 3 (0.04 g). Fraction 3 (0.25 g) was analyzed by ^{13}C NMR and shown to contain a diastereoisomeric mixture of compounds **10** and **11**. Fraction 4 was a mixture of compounds **10**, **11**, and **12**, and fraction 5 contained pure diastereoisomer **12** (0.15 g).

Fraction 3 was rechromatographed (solvent B) and gave pure compound **11** (0.15 g) as an oil and pure compound **10** (0.10 g) as a solid material, which recrystallized from diethyl ether.

Compound **9**: Oil; $[\alpha]^{20}_D$ +68° (*c* 0.5, $CHCl_3$); ^{13}C NMR δ 169.7 (CO ester), 156.6 (CO urethane), 100.6 (C1-Gal), 59.4 (Cα-Thr), 18.1 (Cγ-Thr). Anal. Calcd for $C_{53}H_{55}O_{10}N$: C, 73.52; H, 6.35; N, 1.61. Found: C, 73.62; H, 6.43; N, 1.49.

Compound **10**: White crystals; mp 113–114 °C (from diethyl ether); $[\alpha]^{20}_D$ +5° (*c* 1.0, $CHCl_3$); ^{13}C NMR δ 169.6 (CO ester), 156.5 (CO urethane), 105.0 (C1-Gal), 58.9 (Cα-Thr), 19.1 (Cγ-Thr). Anal. Calcd for $C_{53}H_{55}O_{10}N$: C, 73.52; H, 6.35; N, 1.61. Found: C, 73.64; H, 6.48; N, 1.48.

Compound **11**: Oil; $[\alpha]^{20}_D$ +17.6° (*c* 1.1, $CHCl_3$); ^{13}C NMR δ 169.6 (CO ester), 155.9 (CO urethane), 100.65 (C1-Gal), 59.1 (Cα-Thr), 18.6 (Cγ-Thr). Anal. Calcd for $C_{53}H_{55}O_{10}N$: C, 73.52; H, 6.35; N, 1.61. Found: C, 72.60; H, 6.20; N, 1.53.

Compound **12**: Oil; $[\alpha]^{20}_D$ +9° (*c* 1.25, $CHCl_3$); ^{13}C NMR δ 169.4 (CO ester), 156.0 (CO urethane), 103.0 (C1-Gal), 58.8 (Cα-Thr), 18.0 (Cγ-Thr). Anal. Calcd for $C_{53}H_{55}O_{10}N$: C, 73.52; H, 6.35; N 1.61. Found: C, 72.98; H, 6.33; N, 1.80.

Procedure B. Benzyl chloroformate (0.77 g, 4.5 mmol) was added to a solution of L-allothreonine (0.5 g, 4.2 mL) in water (5 mL) containing sodium bicarbonate (0.85 g) with stirring. After 3 h at room temperature, the reaction mixture was extracted with ether (3 × 10 mL) and acidified with 6 N hydrochloric acid until the pH of the solution was ≃1–2. The aqueous solution was then extracted with ethyl acetate (3 × 20 mL). The organic phase was dried over Na_2SO_4 and concentrated in vacuo to afford *N*-(benzyloxycarbonyl)-L-allothreonine as an oil (0.65 g, 60%): $[\alpha]^{20}_D$ +18° (*c* 2, $CHCl_3$); ^{13}C NMR δ 173.8 (CO acid), 157.2 (CO urethane), 68.9 (Cβ-Thr), 59.9 (Cα-Thr), 18.75 (Cγ-Thr).

The above compound (0.65 g, 2.6 mmol) was allowed to react with benzyl chloride (1 mL) and triethylamine (1 mL) at 75 °C for 1.5 h. Ether (20 mL) and 5% aqueous citric acid (20 mL) were then added to the reaction mixture. The organic layer was removed and the aqueous phase extracted with ether (3 × 10 mL). The combined extracts were washed with 5% aqueous citric acid (10 mL), saturated bicarbonate solution (10 mL), and water (10 mL) and dried over Na_2SO_4. Concentration in vacuo left a white powder which was recrystallized in ether–hexane (yield 0.53 g, 60%): mp 71 °C; $[\alpha]^{20}_D$ +10° (*c* 1, $CHCl_3$); ^{13}C NMR δ 170.3 (CO ester), 156.6 (CO urethane), 67.5 (Cβ-Thr), 59.7 (Cα-Thr), 18.9 (Cγ-Thr).

Finally, benzyl *N*-(benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-benzyl-α- and -β-D-galactopyranosyl)-L-allothreoninate (**9** and **10**) were prepared on a 0.5-mmol scale from the above compound by using the procedure described for compound **6** and **7**. After column chromatography on silica gel, compounds **9** (0.145 g), **10** (0.085 g), and mixture of **9** and **10** (0.25 g) were obtained. An additional crop (0.08 g) of **10** was obtained by recrystallisation of the mixture. The overall yield was 0.42 g (95%).

The physical constants and NMR spectral data of compounds **9** and **10** were identical with those of compounds obtained from D,L-allothreonine by procedure A.

General Conditions Used for the Deprotection of Compounds 6, 7, 9, 10, 11, and 12. Protected galactosyl amino acids (0.5 mmol) dissolved in a mixture of ethanol (20 mL), acetic acid (5 mL), and water (5 mL) were hydrogenolyzed overnight in the presence of 10% palladium-on-charcoal (1.25 g) under 5 bar hydrogen atmosphere. The reaction mixture was filtered, the catalyst thoroughly washed with hot water (2 × 15 mL), and the combined filtrate evaporated in vacuo at 50 °C. Addition of water to the residue, filtration, and concentration were repeated 3 times. The last washing was made with deionized (IRC or Chelex-100) water (10 mL) followed by evaporation in vacuo. The residue was taken off in acetone from which crystallization occurred. Compounds **6**, **7**, **9**, **10**, **11**, and **12** were hence obtained as crystalline white material. The above compounds have been submitted for careful examination by high-field ^1H NMR spectroscopy. Only partial data are reported below for some of them.

O-(α-D-Galactopyranosyl)-D-threonine (**13**): mp 225–227 °C dec; $[\alpha]^{20}_D$ +46° (*c* 1.0, H_2O); ^{13}C NMR, see Table II; ^1H NMR δ 5.09 (d, $J_{1,2}$ = 3.5 Hz, H1), 4.50 (o, $J_{\alpha,\beta}$ = 2.1, $J_{\beta,\gamma}$ = 6.5 Hz, Hβ), 1.34 (d, $C\gamma H_3$).

O-(β-D-Galactopyranosyl)-D-threonine (**14**): mixed with **13**; ^{13}C NMR, see Table II.

O-(α-D-Galactopyranosyl)-L-allothreonine (**15**): mp 232–234 °C dec; $[\alpha]^{20}_D$ +39° (*c* 0.8, H_2O); ^{13}C NMR, see Table IV; ^1H NMR δ 5.14 (d, $J_{1,2}$ = 3.2 Hz, H1), 3.95 (d, $J_{\alpha,\beta}$ = 3.7 Hz, Hα), 4.34 (o, $J_{\beta,\gamma}$ = 6.5 Hz, Hβ), 1.21 (d, $C\gamma H_3$).

O-(β-D-Galactopyranosyl)-L-allothreonine (**16**): mp >240 °C; $[\alpha]^{20}_D$ +3° (*c* 0.8, H_2O); ^{13}C NMR, see Table IV; ^1H NMR δ 4.54 (d, $J_{1,2}$ = 8 Hz, H1), 3.94 (d, $J_{\alpha,\beta}$ = 3.5 Hz, Hα), 4.44 (o, $J_{\beta,\gamma}$ = 6.5 Hz, Hβ), 1.27 (d, $C\gamma H_3$).

O-(α-D-Galactopyranosyl)-D-allothreonine (**17**): mp 225–230 °C dec; $[\alpha]^{20}_D$ +30° (*c* 0.8, H_2O); ^{13}C NMR, see Table IV; ^1H NMR δ 5.13 (d, $J_{1,2}$ = 3.6 Hz, H1), 4.01 (d, $J_{\alpha,\beta}$ = 3.3 Hz, Hα), 4.35 (o $J_{\beta,\gamma}$ = 6.5 Hβ), 1.28 (d, $C\gamma H_3$).

O-(β-D-Galactopyranosyl)-D-allothreonine (**18**): mp 235–237 °C dec; $[\alpha]^{20}_D$ +1° (*c* 1.0, H_2O); ^{13}C NMR, see Table IV; ^1H NMR δ 4.50 (d, $J_{1,2}$ = 8.0 Hz, H1), 3.98 (d, $J_{\alpha,\beta}$ = 3.3 Hz, Hα), 4.38 (o, $J_{\beta,\gamma}$ = 6.6 Hz, Hβ), 1.28 (d, $C\gamma H_3$).

Registry No. **1**, 77943-00-1; **2**, 77943-01-2; **3**, 77943-09-0; **4**, 77943-10-3; **5**, 85995-50-2; **6**, 86023-24-7; **7**, 86023-25-8; **8**, 85995-51-3; **9**, 86023-26-9; **10**, 86023-27-0; **11**, 86023-28-1; **12**, 86023-29-2; **13**, 86023-30-5; **14**, 86023-31-6; **15**, 86023-32-7; **16**, 86023-33-8; **17**, 86023-34-9; **18**, 86023-35-0; D-threoninate hemioxalate, 85995-52-4; benzyl chloroformate, 501-53-1; 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose, 53081-25-7; L-allothreonine, 28954-12-3; *N*-(benzyloxycarbonyl)-L-allothreonine, 85995-53-5; benzyl chloride, 100-44-7; benzyl *N*-(benzyloxycarbonyl)-L-allothreoninate, 84500-41-4.