Enantioselective synthesis of isotopically labelled L-α-amino acids

Preparation of ^{13}C-, ^{18}O- and ^{2}H-labelled L-serines and L-threonines

W.F.J. Karstens, H.J.F.F. Berger, E.R. van Haren, J. Lugtenburg, J.Raap*

Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University

P.O. Box 9502, 2300 RA Leiden, The Netherlands

Summary

[3-^{18}O]-L-serine, [3-^{13}C]-L-serine, [3-^{18}O]-L-threonine, [3,4-$^{13}C_2$]-L-threonine and [3-^{2}H]-L-threonine are prepared from simple commercially available, isotopically enriched starting materials like $H_2{}^{18}O$, [^{13}C]-paraformaldehyde, [$^{13}C_2$]-acetaldehyde and [1-^{2}H]-acetaldehyde. The introduction of the side chain is based on the reaction of the anion of the bislactimether of cyclo-(D-Val-Gly) with a suitable reagent. For serine this is isotopically labelled benzylchloromethylether, whereas for threonine labelled acetaldehyde is used in combination with chlorotitaniumtris[diethylamide], introducing both stereocentres in one single step. The isotopomers of serine and threonine are obtained on the gram scale in good yields and high enantiomeric and diasteriomeric excesses. New syntheses for [^{18}O]-benzylalcohol and isotopically enriched benzylchloromethylether are reported. Following the presented synthetic scheme these amino acids can be labelled at any position or at any combination of positions.

Keywords: [3-^{18}O]-L-serine, [3-^{13}C]-L-serine, [3-^{18}O]-L-threonine, [3,4-$^{13}C_2$]-L-threonine, [3-^{2}H]-L-threonine, [^{18}O]-benzylalcohol, benzylchloromethylether

*To whom correspondence should be addressed. FAX +31 71 274488, E-Mail Raap_J@chem.LeidenUniv.nl

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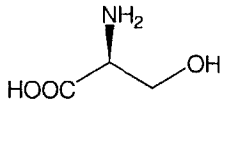
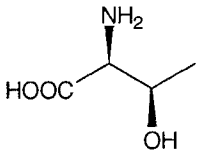
Introduction

In the biological and life sciences the non radioactive ^{18}O-, ^{13}C- and ^{2}H-isotopomers of amino acids are rapidly becoming attractive tools for metabolic tracer studies (1). Moreover, isotopically labelled amino acids are key compounds in the investigation of the active site of proteins by means of various spectroscopic techniques like MAS-NMR and FT-IR (2). In ion channel membrane proteins serine and threonine are expected to facilitate the transport of ions due to the polar character of their side chains. To study the role of threonine in bacteriorhodopsin with FT-IR, [3-^{18}O]-labelled threonine has been incorporated in this transmembrane protein by growing *Halobacterium Salinarium* on a synthetic medium containing the labelled amino acid (3).

In this paper we present convenient syntheses of the isotopomers of L-serine **1a-b** and L-threonine **2a-c** (figure 1) with high stereoselectivity on a preparative scale, starting from simple labelled materials. Also we report new syntheses of ^{18}O-labelled benzylalcohol and of ^{18}O- and ^{13}C-labelled benzylchloromethylether.



1a. [3-^{18}O]-L-serine
1b. [3-^{13}C]-L-serine



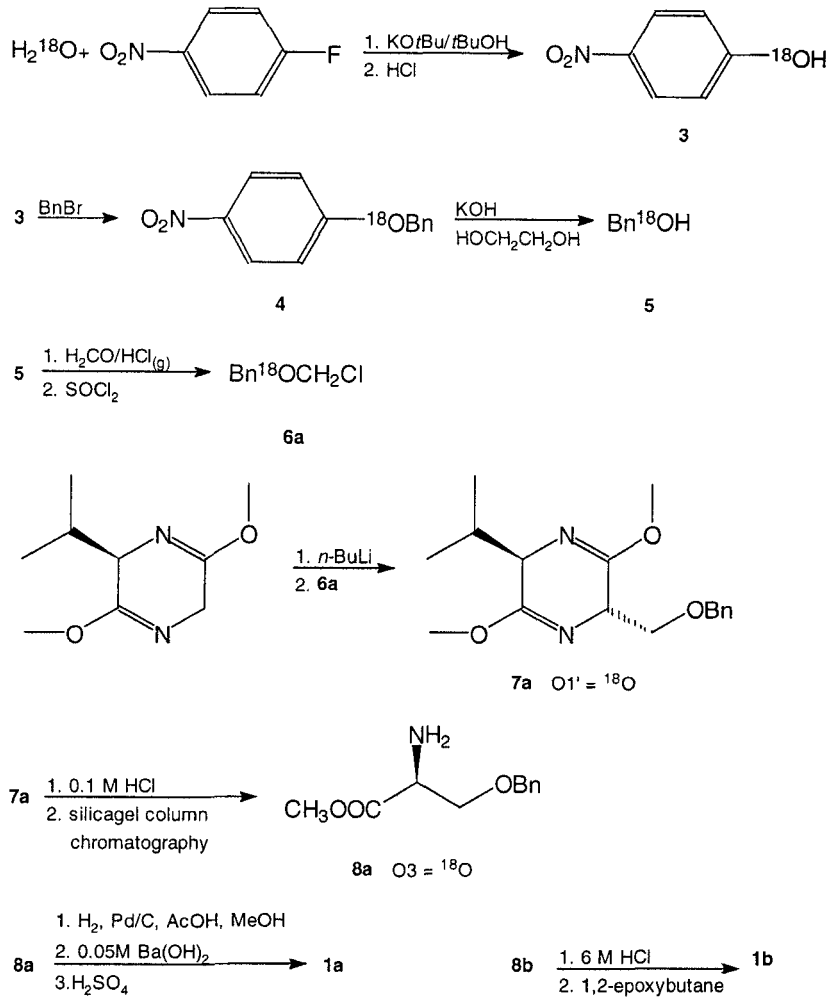
2a. [3-^{18}O]-L-threonine
2b. [3,4-$^{13}C_2$]-L-threonine
2c. [3-^{2}H]-L-threonine

Figure 1: *Synthesized isotopomers of L-serine (**1**) and L-threonine (**2**).*

Synthesis

[3-^{18}O]- and [3-^{13}C]-L-serine

The starting material for [3-^{18}O]-L-serine **1a** (scheme 1) is $H_2{}^{18}O$, that is converted to [^{18}O]-4-nitrophenol **3** (59% ^{18}O), which has previously been used in the synthesis of [^{18}O]-L-tyrosine (4). Compound **3** is converted to [^{18}O]-benzyl-4-nitrophenylether **4** by a S_N2 reaction with benzylbromide under phase transfer conditions (CH_2Cl_2 / 25% aqueous



Scheme 1: *Synthetic sequence for the synthesis of [3-^{18}O]-L-serine (**1a**) and [3-^{13}C]-L-serine (**1b**).*

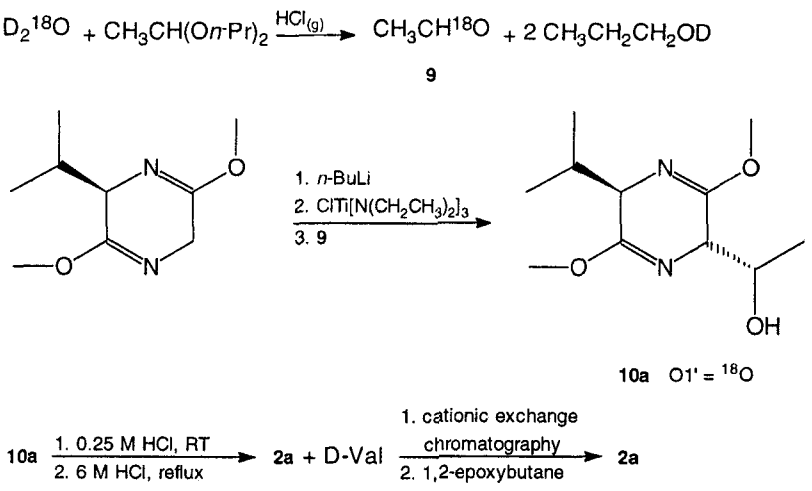
NaOH) with tetra-*n*-butylammoniumbromide as the phase transfer catalyst. Recrystallization from 96% ethanol yields 85% of **4** (59% ^{18}O) which is hydrolysed to [^{18}O]-benzylalcohol **5** (55% ^{18}O) in a 73% yield at 90°C with 10 eq KOH in ethyleneglycol. Compound **6a** has to be synthesized in a water free medium in order to prevent ^{18}O-exchange because of which a new method is developed. Gaseous formaldehyde is led into a solution of **5** in dry THF into which dry HCl gas is injected as a catalyst. The benzyloxymethanol formed *in situ* is converted to the benzylchloromethylether by addition

of thionylchloride to the reaction mixture. The solvent is removed *in vacuo* and the residue distilled under reduced pressure yielding 72% of **6a**. The alkylation of the bislactimether of cyclo-(D-Val-Gly) with **6a** is performed by a procedure described by Nozulak and Schöllkopf (5) for the synthesis of D-serine. The bislactimether of cyclo-(D-Val-Gly) is metallated into its lithium derivative and allowed to react with **6a** yielding the alkylated product **7a**. The product is not purified but hydrolysed directly to the ethyl esters of D-valine and L-serine benzylether, which are separated by silicagel column chromatography yielding 48-58% of **8a** (based on **6a**). The benzyl group of **8a** is not cleaved under acidic aqueous conditions in order to prevent ^{18}O-exchange with water. Instead the benzyl group is removed by catalytic hydrogenation with Pd/C in methanol and acetic acid. The resulting L-serine methyl ester is saponified with a 0.05 M solution of $Ba(OH)_2$ in water, followed by neutralization with dilute sulfuric acid. Filtration and lyophilization yield 83% of **1a** (55% ^{18}O).

The conditions for the reaction of the bislactimether of cyclo-(D-Val-Gly) with ^{13}C-labelled benzyl chloromethylether **6b** and the subsequent hydrolysis to **8b** are the same as described above. The final hydrolysis to [3-^{13}C]-L-serine **1b** is performed with 6 M HCl at reflux temperature giving the HCl salt of **1b**. The product is freed from HCl by treatment with 1,2-epoxybutane yielding 89% of **1b** (99% ^{13}C).

[3-^{18}O]-, [3,4-$^{13}C_2$]- and [3-^{2}H]-L-Threonine

For the synthesis of the isotopomers of L-threonine **2a-c** (scheme 2) we modified the procedure that is described by Schöllkopf *et al.* (6) for the synthesis of D-threonine, in which the bislactimether of cyclo-(L-Val-Gly) is used. In order to be able to introduce both chiral centres in one single step, the anion of cyclo-(D-Val-Gly) is allowed to form a complex with chlorotitaniumtris[diethylamide], before the labelled acetaldehyde is added. The titanium also complexes the oxygen of the added monomeric acetaldehyde creating



Scheme 2: *Enantioselective synthesis of [3-^{18}O]-L-threonine (**2a**).*

a transition state favouring the formation of L-threonine above L-*allo*-threonine. Introduction of the side chain of **2a** requires the use of [^{18}O]-acetaldehyde **9**, which is prepared by the acid catalysed hydrolysis of 1,2-dipropoxyethane with $^{2}H_2{}^{18}O$. First the bislactimether of cyclo-(D-Val-Gly) is metallated into its lithium derivative and chlorotitaniumtris[diethylamide] is added to form the chiral complex. Treatment with **9** yields 58% of the adduct **10a** after purification by means of silica gel column chromatography. Compound **10a** is suspended in 0.25 M HCl and hydrolysed to the methyl esters of D-valine and L-threonine. The concentration of HCl is raised to 6 M and the amino acids are obtained after 4.5 hours at reflux temperature. After the hydrolysis the threonine is separated from valine by means of cationic exchange chromatography with dilute HCl as the eluent. The product is freed from HCl by treatment with 1,2-epoxybutane yielding 88% of **2a** (93% ^{18}O). The ratio L-threonine : L-*allo*-threonine is 15 : 1 as is determined by means of HPLC analysis, and no D-isomers could be detected. The diastereomers can be separated on a preparative scale in order to obtain enantiomeric pure samples (see experimental section). The other two isotopomers of L-threonine **2b-c** (99% ^{13}C and 99% ^{2}H, respectively) are prepared in the same manner, without loss or

scrambling of label, starting with the commercially available [$^{13}C_2$]-acetaldehyde and [1-^{2}H]-acetaldehyde.

Spectroscopic characterization

^{1}H-NMR spectroscopy

The position of the ^{13}C- and ^{2}H-labels have been ascertained by ^{1}H-NMR, which also provides the $^{1}J_{CH}$, $^{2}J_{CH}$ and the $^{3}J_{CH}$ values for the protons neighbouring the ^{13}C-labelled positions (see experimental section). From the ^{1}H-NMR spectrum of **1b** the position of the label is immediately clear from the extra splitting of the signals at 3.96 and 3.94 ppm (H3a and H3b) because of the large $^{1}J_{CH}$ coupling constant ($^{1}J_{C3\text{-}H3a,b}$ = 147 Hz). In the ^{1}H-NMR spectrum of **2b** the positions of the ^{13}C-label are confirmed by two $^{1}J_{CH}$ splittings of the signals at 4.35 (H3) and 1.28 ppm (H4) with $^{1}J_{C3\text{-}H3}$ = 148 Hz and $^{1}J_{C4\text{-}H4}$ = 127 Hz, respectively. From the ^{1}H-NMR spectrum of **2c** the position of the ^{2}H-label is ascertained, because the signal belonging to proton-3 in the natural abundance spectrum is diminished upon substitution with ^{2}H, whereas the signals belonging to H2 and H4 become singlets after deuteration at the H3 position. In the ^{1}H-NMR spectra of the ^{13}C- and ^{2}H-labelled amino acids and their intermediates, no signal of unlabelled compound could be detected, indicating a high degree of isotopic enrichment, *i.e.* ≥99%.

^{13}C-NMR spectroscopy

Further confirmation comes from the ^{13}C-NMR spectra of the products, since the signal of a labelled carbon position is much more intense than a signal coming from a natural abundance carbon position. As well as chemical shift information, this technique provides the $^{1}J_{CC}$ and $^{1}J_{CD}$ values for the carbon at enriched positions. The ^{13}C-NMR spectrum of **1b** shows a strong peak at the chemical shift expected for the 3-carbon (60.9 ppm). The signal coming from C2 (57.0 ppm) is split with 37 Hz ($^{1}J_{CC}$). The spectrum of **2b** displays strong peaks at 65.9 ppm (C3) and at 19.6 ppm (C4), which are split with 38 Hz ($^{1}J_{CC}$).

The small peak at 59.1 ppm (C2) is split with 37 Hz ($^1J_{CC}$) by the 3-carbon. The position of the deuterium label in **2c** is clear because the signal of C3 is split into a triplet with $^1J_{CD}$ = 19 Hz. .

Mass spectroscopy

The use of GCMS allows the calculation of isotopic enrichment of the synthesized amino acids. In order to apply this technique the amino acids had to be converted into more volatile compounds. Following standard procedures the serine and threonine isotopomers are converted into their N,O-bis(trifluoroacetyl) *n*-butyl esters (7) and their N,O,O'-tris(*t*-butyldimethylsilyl) derivatives (8) respectively.

The single-focus EI mass spectrum (70 eV) of the natural abundance derivatized L-serine displays prominent peaks at *m/z* 354 (8%, MH^+), 298 (10%, MH^+- C_4H_8), 240 (9%, M^+- CF_3CO_2), 184 (29%, M^+- (CF_3CO_2 + C_4H_8)) and 139 (100%, M^+- (CF_3CO_2 + $CO_2C_4H_8$)). The mass spectrum of the derivatized [3-^{18}O]-L-serine shows peaks at *m/z* 356, 300, 240, 184, 139. From the peaks at *m/z* 356 and 300 the ^{18}O-incorporation is determined as 55 ± 3%. The mass spectrum of the derivatized [3-^{13}C]-L-serine shows peaks at *m/z* 355, 299, 241, 185, 140. From the peaks at *m/z* 241, 185 and 140 the ^{13}C-incorporation is determined as 99 ± 3%.

The single-focus EI mass spectrum (70 eV) of the natural abundance derivatized L-threonine displays prominent peaks at *m/z* 462 (20%, MH^+), 446 (29%, M^+- CH_3), 404 (63%, M^+- $C(CH_3)_3$), 376 (61%, M^+- (CO + $C(CH_3)_3$)) and 303 (100%, MH^+- (CH_3CHOTBDMS)). The mass spectrum of the derivatized [3-^{18}O]-L-threonine shows peaks at *m/z* 464, 448, 406, 378 and 303. From the peaks at *m/z* 378 and 406 the ^{18}O-incorporation is determined as 93 ± 3%. The mass spectrum of the derivatized [3,4-$^{13}C_2$]-L-threonine shows peaks at *m/z* 464, 448, 406, 378 and 303. From the peaks at *m/z* 378 and 406 the ^{18}O-incorporation is determined as 93 ± 3%. The mass spectrum of the derivatized [3-^{2}H]-L-threonine shows peaks at *m/z* 463, 447, 405, 377 and 303. From the peaks at *m/z* 377 and 405 the ^{2}H-incorporation is determined as 99 ± 3%.

Discussion

In the synthesis of **1a** [^{18}O]-benzylalcohol **5** (55% ^{18}O) is prepared by a nucleophilic aromatic substitution of the benzyloxy group of **4** by a hydroxide ion (mechanism A, figure 2). The reaction conditions are crucial in this step. If the temperature is allowed to become too high the isotope enrichment drops significantly. For instance, at a reaction temperature of 110 °C the ^{18}O-content is as low as 44%. Apparently the ether is also attacked by the hydroxide ion at the benzylic methylene (mechanism B, figure 2) resulting in the formation of unlabelled benzylalcohol. Under the described conditions the selectivity of the reaction is optimal with a reaction time of five hours. The yield of **5** is higher and the label dilution less than in a previously reported synthesis (9).

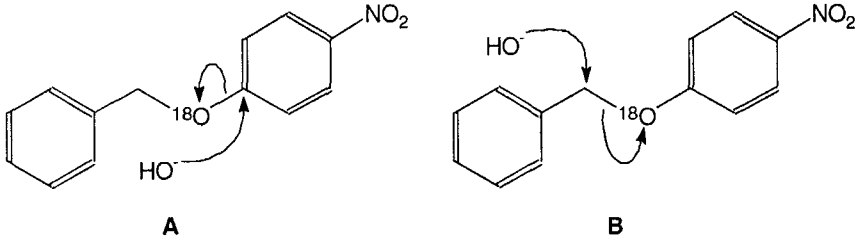


Figure 2: *Preparation of benzylalcohol by a nucleophilic aromatic substitution, yielding labelled (**A**) as well as unlabelled (**B**) product.*

The novel conversion of **5** to **6a** with the use of thionylchloride has the advantage that it is carried out in a water free medium and that it is easily reproduced. The synthesis requires only one equivalent of paraformaldehyde as well as one equivalent of benzylalcohol making this method suitable for the synthesis of both **6a** and **6b**.

In the synthesis of the isotopomers of L-threonine the quality of the acetaldehyde and the chlorotitaniumtris[diethylamide] are of major importance. One must verify by ^{1}H-NMR (see experimental section), that the acetaldehyde is in the monomeric form. If

paraldehyde is used the yield drops dramatically. The quality of the chlorotitanium-tris[diethylamide] is also easily verified by ^{1}H-NMR. If impurities are allowed the diastereomeric excess can become as low as 50%. Therefore the chlorotitaniumtris[diethylamide] is synthesized starting from *n*-butyllithium rather than from metallic lithium (10). We found the latter method hard to reproduce and it gave low yields and a contaminated product.

The spectroscopic characteristics of the isotopomers of L-Ser (**1a-b**) and L-Thr (**2a-c**) have been determined by mass spectroscopy and ^{1}H- and ^{13}C-NMR spectroscopy. The observed fragmentations of the serine and threonine derivatives are in agreement with those presented in literature (11). This confirms the positions of the labels and allows the calculation of the isotopic enrichment. The ^{18}O-incorporation in [3-^{18}O]-L-serine and [3-^{18}O]-L-threonine are determined as 55 ± 3% and 93 ± 3%, respectively. These values are almost the same as those of the enriched starting materials: 59% and 98%, respectively. From the NMR-spectra it is evident that for the ^{13}C- and ^{2}H-labelled products the enrichments are identical with the specifications of the commercial labelled starting materials. This establishes that no isotope dilution of the labelled material has taken place during synthesis.

It should be noted that in the synthesis of L-threonine the byproduct L-*allo*-threonine can be separated from L-threonine by preparative HPLC for spectroscopic analysis. However, if the product is to be used in biological incorporation experiments there is no need for a separation because only the correct diastereomer will be incorporated.

In conclusion, we have developed enantioselective syntheses of L-serine and L-threonine starting from relatively simple labelled compounds that are commercially available. According to these syntheses the side chains of the ß-hydroxy amino acids can be highly enriched with ^{18}O, ^{13}C and ^{2}H. Moreover, by using the ^{15}N- and ^{13}C-labelled bislactimethers of cyclo-(D-Val-Gly) that have previously been used in our group (12), L-serines and L-threonines specifically ^{18}O-, ^{13}C-, ^{15}N- and ^{2}H-enriched in any combination of positions are now available on the gram scale.

Experimental

Instrumentation and analytical procedures

TLC analysis was performed on Merck DC-Alufoliën Kieselgel 60 F_{254} plates (0.2 mm). The elution system BAPW refers to n-butanol/acetic acid/pyridine/water = 15/3/10/12 by volume. Spot detection for amines was established by applying a solution of ninhydrin in ethanol (0.2%), followed by heating. The bislactimether derivatives were detected by treatment with 3 M HCl, followed by ninhydrin. Silicagel column chromatography was performed with Kieselgel 60 from Merck. Resin for cation exchange chromatography, AG 50W-X8, was obtained from Biorad.

^{1}H-NMR spectra were recorded on a Bruker WM-300 or a Jeol NM FX-200 in C^2HCl_3 or ^2H_2O using TMS (δ 0.00 ppm) and H^2HO (δ 4.9 ppm) as internal standards. ^{13}C-NMR spectra were recorded on a Bruker WM-300 at 75 MHz using C^2HCl_3 (δ 77.0 ppm) as internal standard.

The mass spectra of compounds **4** and **5** were recorded on a Kratos 9/50-mass spectrometer. In order to determine the isotopic enrichment by GCMS analysis the serine and threonine isotopomers were first converted by standard procedures into their N,O-bis(trifluoroacetyl) *n*-butyl esters (7) and N,O,O-tris(tert-butyldimethylsilyl) derivatives (8), respectively. The amino acid derivatives were separated on a Hewlett Packard 438 gas chromatograph connected to a Finnigan MAT ITD 700 mass spectrometer. The mass spectrometer was connected to an IBM AT personal computer with ITD software 3.0 installed in order to control the automatic gain of the mass spectrometer and to acquire and integrate signals. For the serine derivatives the gas chromatograph was equipped with a 50 m CP-Sil 5 capillary column that was programmed from 100 to 200°C at 3°C/min. The threonine derivatives were separated on a 25 m SE 30 capillary column, which was programmed from 100 to 300°C at 8°C/min.

The enantiomeric purity of the synthesized compounds was determined by HPLC using a Pharmacia LKB gradient delivery system, a Rheodyne injection valve fitted with a 5 µm loop and a Spectro-Vision FD-300 fluorescence detector. In order to detect a signal the

amino acids were first derivatized to fluorescent compounds using benzene-1,2-dicarbaldehyde (Janssen) and N-acetyl-cysteine (Aldrich) (13). The excitation and detection wavelengths were 360 and 405 nm, respectively.

To obtain enantiomeric pure samples for the characterization by means of spectroscopic techniques threonine and *allo*-threonine could be separated by preparative HPLC. Precolumn derivation of the threonines to O-Fmoc-threonines according to a procedure described by Paquet (14) for serines was followed by a preparative HPLC separation. The used column was a 250 x 20 mm C-18 10 µm reverse phase column (Pharmacia). The eluent (methanol/0.1% trifluoroacetic acid in water = 6/4 (v/v)) flows at a rate of 10 ml/min. The threonine derivatives were injected at a concentration of 2.6 mg/ml and not more than 0.5 ml was injected per separation. The excitation and detection wavelengths were 260 and 310 nm, respectively. By an automated, computerized procedure 145 mg (0.42 mmol) of Fmoc-threonine was purified. Subsequent deprotection with 2% diethylamine in DMSO gave optically pure L-threonine.

Materials

The ^{18}O-labelled water was purchased from ICON, New York. The [^{13}C]-paraformaldehyde, the doubly labelled [$^{13}C_2$]-acetaldehyde and [1-^{2}H]-acetaldehyde, were purchased from Cambridge Isotope Laboratories, INC. The acetaldehydes were checked for the presence of para-acetaldehyde by means of ^{1}H-NMR. If para-acetaldehyde (δ = 5.0 ppm, quartet and 1.4 ppm, doublet) was found, the acetaldehyde was freshly distilled before use. If in the ^{1}H-NMR spectrum of chlorotitaniumtris[diethylamine] impurities were found it was also freshly distilled. (*2R*)-2,5-dihydro-2-isopropyl-3,6-dimethoxypyrazine was purchased from Merck. In all experiments distilled, dry solvents were used. Diethylether was dried over phosphorous pentoxide for one day and then distilled. The ether was stored over sodium wire. Ether refers to diethylether and petroleum ether refers to low boiling petroleum ether 40-60. Tetrahydrofuran was refluxed with $LiAlH_4$ for several hours and always freshly distilled before use.

[^{18}O]-Benzyl-4-nitrophenylether (4): To a solution of 6.03 g (43 mmol) of [1-^{18}O]-4-nitrophenol **3** (59% ^{18}O) and 14.6 g (86 mmol) of benzylbromide in 60 ml of methylenechloride, 60 ml of 25%-NaOH and a catalytic amount of Bu_4NBr were added. The resulting slurry was heated to reflux temperature. After 5 h of reflux all solid was dissolved indicating completion of the reaction. After cooling 250 ml of water was added and the layers were allowed to separate. The organic layer was washed with 75 ml of 1 M NaOH and 75 ml of water. After drying over Na_2SO_4 the solvent was evaporated *in vacuo* and recrystallisation from 96%-ethanol 8.35 g (36 mmol, 84%) of **4** was obtained as colourless crystals. From the mass spectrum the ^{18}O incorporation was calculated: 59 ± 3%.

^{1}H-NMR (200 MHz, C^2HCl_3): δ(ppm) 8.21 (d, 2H, $^3J_{HH}$ = 9.2 Hz, H3, H5), 7.41 (s, 5H, C_6H_5), 7.03 (d, 2H, $^3J_{HH}$ = 9.2 Hz, H2, H6), 5.17 (s, 2H, $ArCH_2$).

EI-MS: m/z 65 (53%, $C_5H_5^+$), 91 (100%, $C_7H_7^+$), 229 (10%, M^+), 231 (15%, M^+).

[^{18}O]-Benzylalcohol (5): Under nitrogen atmosphere a solution of 2.00 g (8.7 mmol) of **4** and 4.9 g (87 mmol) of KOH in 20 ml of ethyleneglycol was heated to 90°C. After 5 h TLC analysis (ether/petroleum ether = 1/1 (v/v)) indicated completion of the reaction, and the mixture was allowed to attain room temperature. Water was added and the pH lowered to pH=9 with aqueous 6M HCl. The solution was extracted four times with 100 ml of ether and the combined organic layers were dried over $MgSO_4$. After removal of the solvent *in vacuo* and purification by silicagel chromatography (ether/petroleum ether = 1/1 (v/v)) the yield of **5** was 0.69 g (6.3 mmol, 72%). The ^{18}O-incorporation was 55 ± 3%.

^{1}H-NMR (200 MHz; C^2HCl_3): δ(ppm) 7.35 (s, 5H, C_6H_5)), 4.68 (s, 2H, CH_2), 1.79 (s, 1H, OH)

EI-MS: m/z 77 (28%, $C_6H_5^+$), 79 (61%, $C_6H_7^+$), 91 (100%, $C_7H_7^+$), 107 (38%, M^+), 108 (25%, MH^+), 109 (46%, M^+), 110 (30%, MH^+).

[^{18}O]-Benzylchloromethylether (6a): A two-necked reaction flask, containing 1.44 g (48 mmol) of paraformaldehyde, was connected to a gasinlet of a three-necked reaction - flask, containing a solution of 3.48 g (32 mmol) of **5** in 10 ml of dry THF. Using a syringe 10 ml of dry HCl-gas was injected into this solution. The paraformaldehyde was heated to 120°C under a nitrogen flow, which was led through the stirred solution. After all the paraformaldehyde had disappeared stirring was maintained at room temperature for 1 hour. The solution was cooled to 5°C and 3.84 g (32 mmol) of thionylchloride was slowly added. The solvent was removed *in vacuo* and the residue distilled under reduced pressure yielding 3.61 g of **6a** (23 mmol, 72%, based on **5**). B.p. 53-63°C (0.3 Torr).
^{1}H-NMR (200 MHz; C^2HCl_3): δ(ppm) 7.36 (s, 5H, C_6H_5), 5.53 (s, 2H, OCH_2Cl), 4.75 (s, 2H, $PhCH_2O$)

[^{13}C]-Benzylchloromethylether (6b): 1.00 g (32 mmol) of [^{13}C]-paraformaldehyde and 3.48 g (32 mmol) of benzylalcohol yielded 3.35 g of **6b** (21 mmol, 67%, based on [^{13}C]-paraformaldehyde), using the same method as described above.
^{1}H-NMR (200 MHz, C^2HCl_3): δ(ppm) 7.36 (s, 5H, C_6H_5), 5.53 (d, 2H, $^1J_{CH}$ = 176 Hz, OCH_2Cl), 4.75 (d, 2H, $^3J_{CH}$ = 4.1 Hz, $PhCH_2$)

[1'-^{18}O]-(*2R,5S*)-5-Benzyloxymethyl-2,5-dihydro-2-isopropyl-3,6-dimethoxypyrazine (7a): Under nitrogen atmosphere a solution of 1.82 g (9.9 mmol) (*2R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine in 15 ml of dry THF in a three-necked round-bottomed reaction flask was prepared and cooled to -70°C. A volume of 6.2 ml of a 1.6 M *n*-butyllithium solution in *n*-hexane was slowly injected and after stirring for 15 min. a solution of 1.30 g (8.2 mmol) of **6a** in 4.5 ml of dry THF was added. Stirring was maintained for 5 hours at -70°C and the solution was allowed to obtain room temperature. The solvent was removed *in vacuo* and 7.5 ml of phosphate buffer (pH=7) was added to the residue. The water layer was extracted four times with 100 ml of ether and the

combined organic layers were dried over $MgSO_4$. Removal of the solvent *in vacuo* yielded 1.13 g of crude **7a**. Compound **7a** could be purified by silicagel chromatography (ethyl acetate/petroleum ether 40-60 = 3/7 (v/v)) yielding 61% pure **7a** but it could also be used without further purification in the next step.

^{1}H-NMR (300 MHz, C^2HCl_3): δ(ppm) 7.29 (m, 5H, C_6H_5), 4.54 (s, 2H, $PhCH_2O$), 4.09 (m, 1H, $^3J_{HH}$ = 3.7 Hz, $^3J_{HH}$ = 2.9 Hz, $^5J_{HH}$ = 3.5 Hz, H5), 4.02 (t, 1H, $^3J_{HH}$ = 3.3 Hz, $^5J_{HH}$ = 3.5 Hz, H2), 3.84 (dd, 1H, $^2J_{HH}$ = 9.4 Hz, $^3J_{HH}$ = 3.7 Hz, H1'a), 3.74 (dd, 1H, $^2J_{HH}$ = 9.4 Hz, $^3J_{HH}$ = 2.9 Hz, H1'b), 3.72, 3.71 (2s, 6H, $2OCH_3$), 2.31 (m, 1H, $^3J_{HH}$ = 6.8 Hz, $^3J_{HH}$ = 3.3 Hz, ^{i}PrCH), 1.07, 0.70 (2d, 6H, $^3J_{HH}$ = 6.8 Hz, 2^iPrCH$_3$)

[1'-^{13}C]-(*2R,5S*)-5-Benzyloxymethyl-2,5-dihydro-2-isopropyl-3,6-dimethoxypyrazine (7b): Following the same method as described above 1.82 g (9.9 mmol) of (*2R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine and 1.30 g (8.2 mmol) of **6b** were converted to 1.14 g of crude **7b**, which was used unpurified in the next step. A pure sample could be obtained in the same manner as described for **7a**.

^{1}H-NMR (300 MHz, C^2HCl_3): δ(ppm) 4.54 (d, 2H, $^3J_{CH}$ = 4.6 Hz, $PhCH_2O$), 4.09 (m, 1H, $^3J_{HH}$ = 2.9 Hz, $^3J_{HH}$ = 3.7 Hz, $^5J_{HH}$ = 3.5 Hz, H5), 4.02 (t, 1H, $^3J_{HH}$ = 3.3 Hz, $^5J_{HH}$ = 3.5 Hz, H2), 3.84 (ddd, 1H, $^1J_{CH}$ ≈ 150 Hz, $^2J_{HH}$ = 9.4 Hz, $^3J_{HH}$ = 3.7 Hz, H1'a), 3.74 (ddd, 1H, $^1J_{CH}$ ≈ 150 Hz, $^2J_{HH}$ = 9.4 Hz, $^3J_{HH}$ = 2.9 Hz, H1'b), all other signals were the same as observed for **7a**.

[3-^{18}O]-O-Benzyl-L-serine methyl ester (8a): To 1.13 g of crude **7a** 200 ml of 0.1 M HCl was added. The remaining suspension was stirred for 2h at 40°C, until a clear solution was obtained and TLC analysis indicated completion (BAPW). The solution was neutralized using 25% ammonia and concentrated *in vacuo* to 50 ml.. The pH was raised to pH=10 with 25% ammonia and saturated with NaCl followed by extraction with four times 100 ml of ether. The combined organic layers were dried over $MgSO_4$ and the

solvent was removed *in vacuo*. The crude product was subjected to silicagel chromatography (ethanol/ether = 5/95 (v/v)), yielding 0.83 g of **8a** (3.94 mmol, 48% based on **6a**).
^{1}H-NMR (200 MHz, C^2HCl_3): δ(ppm) 7.32 (s, 5H, C_6H_5), 4.54 (s, 2H, $PhCH_2$), 3.73 (s, 3H, OCH_3), 3.71 (d, 2H, $^3J_{HH}$ = 5.0 Hz, H3), 3.68 (d, 1H, $^3J_{HH}$ = 5.0 Hz, H2), 1.71 (s, 2H, NH_2)

[3-^{13}C]-O-Benzyl-L-serine methyl ester (8b): Starting with 1.14 g of crude **7b** the same procedures as mentioned above yielded 1.00 g of **8b** (4.76 mmol, 58% based on **6b**)
^{1}H-NMR (200 MHz, C^2HCl_3): δ(ppm) 4.54 (d, 2H, $^3J_{CH}$ = 4.1 Hz, $PhCH_2$), 3.71 (dd, 2H, $^1J_{CH}$ = 144 Hz, $^3J_{HH}$ = 5.0 Hz, H3), 1.71 (s, 2H, NH_2), all other NMR data were the same as described for **8a**.

[3-^{18}O]-L-Serine (1a): To a solution of 0.98 g (4.6 mmol) of **8a** in a 20 ml of a degassed mixture of acetic acid and methanol (5/95 (v/v)) was added a catalytic amount of Pd/C and H_2 gas was led through at atmospheric pressure while stirring. After 5h a new portion of catalyst was added. TLC analysis (BAPW) indicated completion of the reaction after 12h. The reaction mixture was filtrated and the solvent removed *in vacuo*. The residue was dissolved in 70 ml of 0.05 M $Ba(OH)_2$ and after 1h at room temperature the mixture was neutralized with a 0.5 M H_2SO_4 solution. Filtration and lyophilization yielded 0.41 g (3.8 mmol, 83%) of **1a** (55% ^{18}O).
^{1}H-NMR (300 MHz, ^2H_2O): δ(ppm) 3.96 (dd, 1H, $^2J_{HH}$ = 12.3 Hz, $^3J_{HH}$ = 3.9 Hz, H3a), 3.94 (dd, 1H, $^2J_{HH}$ = 12.3 Hz, $^3J_{HH}$ = 5.5 Hz, H3b), 3.84 (dd, 1H, $^3J_{HH}$ = 5.5 Hz, $^3J_{HH}$ = 3.9 Hz, H2)
^{13}C-NMR (75 MHz, ^2H_2O): δ(ppm) 173.0 (C1), 60.8 (C3), 57.0 (C2)

[3-^{13}C]-L-Serine (1b): A solution of 2.19 g (10.4 mmol) of **9b** in 100 ml of 6M HCl was refluxed for 3h, after which TLC analysis (BAPW) indicated completion. The solution was evaporated to dryness *in vacuo* and coevaporated with water until all excess HCl was removed. The residue was dissolved in 45 ml of EtOH and 20 ml of 1,2-epoxybutane

was added. Refluxing for 1 hour followed by cooling and centrifugation of the suspended serine yielded 0.99 g (9.3 mmol, 89%) of **1b** (99% ^{13}C).

^{1}H-NMR (300 MHz, ^2H_2O): δ(ppm) 3.96 (ddd, 1H, $^1J_{CH}$ = 147 Hz, $^2J_{HH}$ = 12.3 Hz, $^3J_{HH}$ = 3.9 Hz, H3a), 3.94 (ddd, 1H, $^1J_{CH}$ = 147 Hz, $^2J_{HH}$ = 12.3 Hz, $^3J_{HH}$ = 5.5 Hz, H3b), 3.84 (dt, 1H, $^2J_{CH}$ = 3.9 Hz, $^3J_{HH}$ = 5.5 Hz, $^3J_{HH}$ = 3.9 Hz, H2)

^{13}C-NMR (75 MHz, ^2H_2O): δ(ppm) 173.0 (s, C1), 60.9 (strong peak, s, C3), 57.0 (d, $^1J_{CC}$ = 37 Hz, C2)

[^{18}O]-acetaldehyde (9): All the glass was thoroughly dried before use. A 25 ml reaction flask was equipped with a septum, vigreux, thermometer, cooler and receiver. A safety trap was installed behind the receiver. Under nitrogen atmosphere, 3.2 ml of dry HCl-gas was injected using a syringe in the water layer of a mixture of 10.45 g (72 mmol) of 1,1-dipropoxyethane and 1.11 g (55.5 mmol) of [^{18}O]-deuteriumoxide. The stirred mixture was heated to 80°C while the safety trap and the receiver containing 3.15 g of dry THF were cooled to -78°C. The product was collected at the temperature of 21 to 26°C. Yield 2.22 g (48.3 mmol, 87%). The acetaldehyde solution was stored at -20°C until used.

^{1}H-NMR (100 MHz, THF, C^2HCl_3): δ(ppm) 9.82 (q, 1H, $^3J_{HH}$ = 2.9 Hz, H2), 3.71 (m, THF), 2.20 (d, 3H, $^3J_{HH}$ = 2.9 Hz, H1), 1.89 (m, THF)

Chlorotitaniumtris[diethylamide]: Under nitrogen atmosphere and temperature below -20°C 12.0 g (165 mmol) of anhydrous diethylamine was added dropwise to 100 ml of 1.6 M *n*-butyllithium in *n*-hexane in a 250 ml three necked reaction flask. After stirring for 30 min. at -10°C the mixture was allowed to attain room temperature and the solvent was removed from the white suspension by leading through N_2 gas. The white residue was dissolved in 100 ml of dry ether and the temperature lowered to -50°C. Under vigorous stirring a solution of 10.2 g (53.8 mmol) of $TiCl_4$ in 50 ml of toluene was slowly added, affording a brown precipitate. After refluxing for 2 hours a dark brown solution and a precipitate of white lithium salts were obtained. The ether was removed by leading

through N_2 and 100 ml of dry toluene was added. The precipitate was allowed to settle for 2 hours and the solution was decanted and filtrated under nitrogen atmosphere. The residue was washed with 20 ml of dry toluene and also decanted and filtered. The combined filtrates were evaporated *in vacuo*, affording a dark red syrup. After distillation of the residue under reduced pressure 10.71 g of $ClTi[N(C_2H_5)_2]_3$ (35.7 mmol, 67%) as a dark red liquid, crystallizing upon storage at -20°C. B.p. 101°C (0.07 Torr).

^{1}H-NMR (100 MHz, C^2HCl_3): δ(ppm) 3.64 (q, 4H, $^3J_{HH}$ = 6.9 Hz, $2CH_2$), 1.11 (t, 6H, $^3J_{HH}$ = 6.9 Hz, $2CH_3$)

[1'-^{18}O]-(*2R,5S,1'R*)-2,5-dihydro-5-(1'-hydroxyethyl)-2-isopropyl-3,6-dimethoxypyrazine (10a): Under nitrogen atmosphere 5.6 ml of a 1.6 M solution of *n*-butyllithium in *n*-hexane was added to a stirred solution of 1.50 g (8.2 mmol) of (*2R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine in 25 ml of dry THF at -70°C in a 100 ml three necked reaction flask. After stirring had been continued for 15 min. at -70°C, 9.0 ml of a 1.0 M solution of $ClTi[N(C_2H_5)_2]_3$ in *n*-hexane was slowly injected and 45 min later 1.0 g of an [^{18}O-]-acetaldehyde solution in dry THF of 8.98 mmol per gram of solution diluted with 15 ml of THF was added dropwise to the red reaction mixture and the flask was stored overnight at -78°C. After hydrolysis the mixture with 100 ml of a 3 M phosphate buffer at pH=7, the mixture was *allo*wed to obtain room temperature. 50 ml of ether was added and the water layer was extracted three times with 50 ml of ether. The combined organic layers were dried over $MgSO_4$. The solvent was removed *in vacuo* and pure **10a** (1.09 g, 58% based on [^{18}O]-acetaldehyde) was obtained by silicagel chromatography (ether/petroleum ether = 1/1 (v/v)) as the eluent, R_f=0.3. Also unconverted (*2R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (0.16 g, 0.9 mmol) could be regained, R_f=0.7.

^{1}H-NMR (300 MHz, C^2HCl_3): δ(ppm) 4.13 (m, 1H, $^3J_{HH}$ = 6.4 Hz, $^3J_{HH}$ = 3.6 Hz, H1'), 3.99 (t, 1H, $^3J_{HH}$ = 3.6 Hz, $^5J_{HH}$ = 3.6 Hz, H2), 3.91 (t, 1H, $^3J_{HH}$ = 3.6 Hz, $^5J_{HH}$ = 3.6 Hz, H5), 3.75, 3.72 (2s, 6H, $2OCH_3$), 2.27 (m, 1H, $^3J_{HH}$ = 6.8 Hz, $^3J_{HH}$ = 3.6 Hz, ^{i}Pr-CH), 1.58 (s, 1H, OH), 1.29 (d, 3H, $^3J_{HH}$ = 6.4 Hz, H2'), 1.05, 0.71 (2d, 6H, $^3J_{HH}$ = 6.8 Hz, 2^iPr-CH_3)

^{13}C-NMR (75 MHz, C^2HCl_3): δ(ppm) 165.4, 162.0 (C3,C6), 68.8 (C1'), 61.0 (C2), 60.5 (C5), 52.6 ($2OCH_3$), 31.9 (^{i}PrCH), 19.7 (C2'), 19.0, 16.7 (2^iPrCH$_3$)

[1',2'-$^{13}C_2$]-(*2R,5S,1'R*)-2,5-dihydro-5-(1'-hydroxyethyl)-2-isopropyl-3,6-dimethoxypyrazine (10b): Using the same methods and quantities as above **10b** could be synthesized from [$^{13}C_2$]-acetaldehyde.

^{1}H-NMR (300 MHz, C^2HCl_3): δ(ppm) 4.13 (dm, 1H, $^1J_{CH}$ = 150 Hz, $^3J_{HH}$ = 6.4 Hz, $^3J_{HH}$ = 3.6 Hz, H1'), 3.91 (m, 1H, $^3J_{HH}$ = 3.6 Hz, $^5J_{HH}$ = 3.6 Hz, $^2J_{CH}$ + $^3J_{CH}$ = 7.7 Hz, H5), 1.29 (ddd, 3H, $^3J_{HH}$ = 6.4 Hz, $^2J_{CH}$ = 4.4 Hz, $^1J_{CH}$ = 126 Hz, H2'), all other signals were the same as reported for **10a**.

^{13}C-NMR (75 MHz, C^2HCl_3): δ(ppm) 68.8 (strong peak, d, $^1J_{CC}$ = 39 Hz, C1'), 60.5 (d, $^1J_{CC}$ = 39 Hz, C5), 19.7 (strong peak, d, $^1J_{CC}$ = 39 Hz, C2'), the other NMR data were the same as observed for **10a**.

[1'-^{2}H]-(*2R,5S,1'R*)-2,5-dihydro-5-(1'-hydroxyethyl)-2-isopropyl-3,6-dimethoxypyrazine (10c): Using the same methods and quantities as above **10c** could be synthesized from [1-^{2}H]-acetaldehyde.

^{1}H-NMR (300 MHz, C^2HCl_3): δ(ppm) 4.13 (no signal, H1'), 3.91 (d, 1H, $^5J_{HH}$ = 3.6 Hz, H5), 1.29 (s, 3H, H2'), all other signals were the same as observed for **10a**.

^{13}C-NMR (75 MHz, C^2HCl_3): δ(ppm) 68.8 (t, $^1J_{CD}$ = 22 Hz, C1'), the other peaks were the same as described for **10a**.

[3-^{18}O]-L-Threonine (2a): 2.35 g (10.2 mmol) of **10a** was suspended in 80 ml of 0.25 M HCl and stirred for one hour at room temperature to obtain a mixture of the methyl esters of D-valine and L-threonine. To this mixture 27.5 ml of conc. HCl was added and the mixture refluxed for 4.5 hours until TLC-analysis indicated completion ($CHCl_3$/MeOH/NH3(aq) = 10/10/1 (v/v/v)). R_fThr = 0.2. R_fVal = 0.3. The reaction mixture was filtrated over a GF/A Whatman glass fiber filter. The solvent was removed *in vacuo*

and the residue was coevaporated three times with 75 ml of H_2O to remove the excess of hydrochloric acid. The amino acids were separated by cationic ion exchange with a AG-50W X8 resin in the protonated form, with 0.1 M HCl as the eluent. After removal of the solvent *in vacuo* and three times coevaporation with water, the residue was dissolved in 45 ml of EtOH and 19.2 ml of 1,2-epoxybutane was added. Refluxing for 1 hour followed by cooling to room temperature and centrifugation of the suspended threonine yielded 1.08 g (8.92 mmol, 88%) [3-^{18}O]-L-threonine (93% ^{18}O).

^{1}H-NMR (300 MHz, ^2H_2O): δ(ppm) 4.36 (m, 1H, $^3J_{HH}$ = 3.9 Hz, $^3J_{HH}$ = 6.6 Hz, H3), 3.90 (d, 1H, $^3J_{HH}$ = 3.9 Hz, H2), 1.31 (d, 3H, $^3J_{HH}$ = 6.6 Hz, H4)

^{13}C-NMR (75 MHz, ^2H_2O): δ(ppm) 171.2 (C1), 66.4 (C3), 58.6 (C2), 18.4 (C4)

[3,4-$^{13}C_2$]-L-Threonine (2b): Following the same quantities and procedures as described above, **10b** was converted to **2b** (99% ^{13}C).

^{1}H-NMR (300 MHz, ^2H_2O): δ(ppm) 4.35 (dm, 1H, $^3J_{HH}$ = 3.9 Hz, $^3J_{HH}$ = 6.6 Hz, $^1J_{CH}$ = 148 Hz, $^2J_{CH}$ = 2.4 Hz, H3), 3.95 (m, 1H, $^3J_{HH}$ = 3.9 Hz, $^3J_{CH}$ = 3.5 Hz, $^2J_{CH}$ = 1.8 Hz, H2), 1.28 (ddd, 3H, $^3J_{HH}$ = 6.6 Hz, $^2J_{CH}$ = 4.5 Hz, $^1J_{CH}$ = 127 Hz, H4)

^{13}C-NMR (75 MHz, ^2H_2O): δ(ppm) 171.2 (s, C1), 65.9 (strong peak, d, $^1J_{CC}$ = 37.8 Hz, C3), 59.1 (d, $^1J_{CC}$ = 36.7 Hz, C2), 19.6 (strong peak, d, $^1J_{CC}$ = 37.8 Hz, C4)

[3-^{2}H]-L-Threonine (2c): Following the same quantities and procedures as described above, **10c** was converted to **2c** (99% ^{2}H).

^{1}H-NMR (300 MHz, ^2H_2O): δ(ppm) 4.00 (s, 1H, H2), 1.32 (s, 3H, H4)

^{13}C-NMR (75 MHz, ^2H_2O): δ(ppm) 65.6 (t, $^1J_{CD}$ = 19 Hz, C3), all other signals were the same as reported for **2a**.

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