Pergamon

Tetrahedron: *Asymmetry* 12 (2001) 2783–2787

TETRAHEDRON: *ASYMMETRY*

Enzymatic synthesis of 2-deoxy-β-glucosides and stereochemistry of β-glycosidase from *Sulfolobus solfataricus* on glucal†

Antonio Trincone,[a,*] Edoardo Pagnotta,[a] Mosè Rossi,[b,c] Marialuisa Mazzone[c] and Marco Moracci[c]

[a]*Istituto per la Chimica di Molecole di Interesse Biologico CNR, Via Campi Flegrei, ex-Comprensorio Olivetti Arco Felice, Naples, Italy*

[b]*Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II, Via Mezzocannone 16, I-80134 Naples, Italy*

[c]*Institute of Protein Biochemistry and Enzymology CNR, Via Marconi 10, I-80125 Naples, Italy*

Received 14 September 2001; accepted 29 October 2001

Abstract—The hyperthermophilic member of family 1 of the glycosyl hydrolases, the β-glycosidase from the archaeon *Sulfolobus solfataricus* (Ssβ-gly), has been used for an efficient synthesis of β-2-deoxyglucosides and for stereochemical studies of the reactions of glucal in the presence of alkyl and pyranosidic acceptors. Protonation of the double bond of glucal resulting in the equatorially disposed proton was observed and an indication of the protonating amino acid in the active site was obtained by the use of a mutant enzyme. The regioselectivity in the formation of β-2-deoxyglucosides of pyranosidic acceptors is different from that reported for mesophilic biocatalysts.

1. Introduction

One of the most hyperthermophilic glycosyl hydrolases known is the β-glycosidase from the archaeon *Sulfolobus solfataricus* (Ssβ-gly) which shows optimal activity at temperatures over 85°C, remarkable thermostability (half-life at 75°C: >24 h) and activity in the presence of high concentrations of organic compounds. Ssβ-Gly possesses broad hydrolytic substrate specificity and can also synthesize different β-D-glycosides efficiently by transglycosylation using an activated donor as the source of the carbohydrate moiety.[1,2] A high preference for transfer to primary hydroxyl groups is always observed for glucose, galactose and fucose to different diols, triols and β-pyranosidic acceptors.[2] The enzyme produces glycosides much less efficiently by reverse hydrolysis reaction.[3] Ssβ-Gly modified by site-directed mutagenesis at the nucleophile (E387) of the active site has recently been characterized[4] as *glycosynthase* and used for the synthesis of unusual β-(1-3)-β-(1-6) branched bioactive oligosaccharides.[5] These results confirmed the existence of different key characteristics in terms of regioselectivity for this biocatalyst if compared to the same reaction catalyzed by mesophilic representatives. Although this difference does not claim that it is directly linked to the thermophilic nature of the enzyme, it is evident that exploration of the biodiversity can lead to the discovery of enzymes of this class with novel and useful attributes for synthesis.

The stereochemical study of the reactions of glycosidases with 1,2-unsaturated enol ether derivatives of sugars provided insights into the specificity and functioning of this class of biocatalysts.[6] These substrates operate both as glycosidase inhibitors and as donors in the synthesis of 2-deoxyglucosides.[6–9] The chemistry of glycal inhibition consists of its functioning as a substrate, in fact these enol ethers are hydrated by the enzyme with the formation and accumulation of a 2-deoxyglycosyl enzyme intermediate.

During D-galactal hydration by β-galactosidase from *E. coli*, proton delivery results from the action of the protonated catalytic nucleophile in the active site of the enzyme and occurs to the bottom face of the glycal ring, forming the 2-deoxyglycosyl enzyme intermediate.[7–11] Slow hydrolysis of the latter can lead to the formation of 2-deoxysugar or 2-deoxyglycosides of other acceptors present in the reaction mixture (Fig. 1).

^{1}H NMR analysis of the products of hydrolysis using the 1,2-unsaturated enol ether derivatives and β-galactosidase from *E. coli* lead to the proposed mechanism

* Corresponding author. Tel.: +39 081-8675095; fax: +39 081-8041770; e-mail: atrincone@icmib.na.cnr.it

† This paper is dedicated to the memory of Professor Guido Sodano.

0957-4166/01/$ - see front matter
PII: S0957-4166(01)00491-8

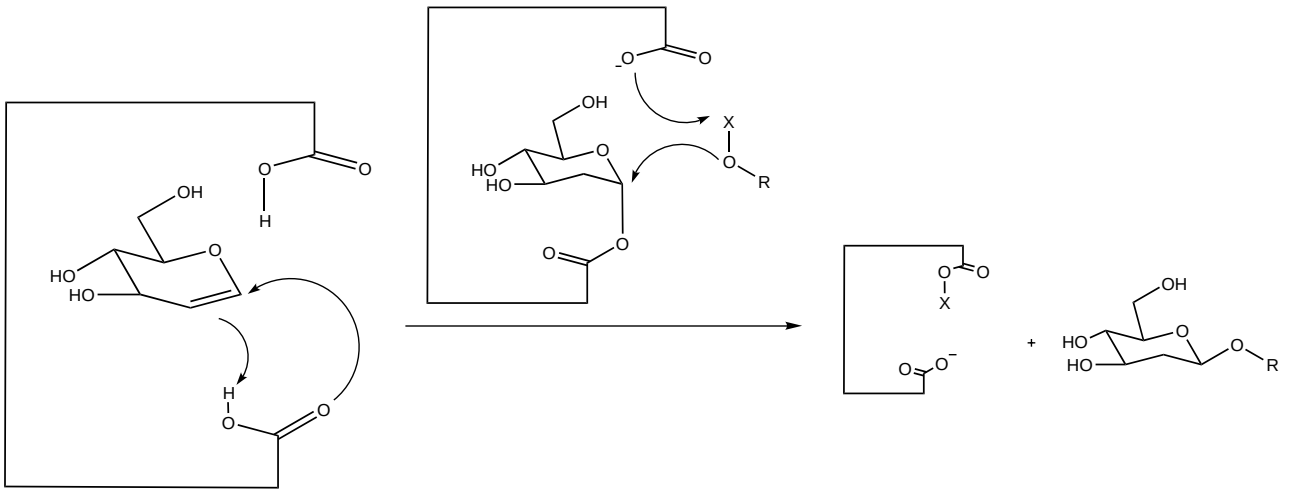

Figure 1. Chemistry of glucal action with β-glycosyl hydrolases (X = H or D).

in Fig. 1 in which the 2-deoxyglycosyl enzyme intermediate arises from concerted action of the protonated catalytic nucleophile (Glu537) across the double bond. A series of similar outcomes have been observed for other enzymes.[7]

Owing to the occurrence of β-2-deoxyglycosyl moieties in many antibiotics, the anomerically selective enzymatic synthesis of β-2-deoxy glycosides is a very interesting approach since their chemical synthesis is a problem of current interest which has been resolved by using stereodirecting auxiliary groups equatorially disposed at C(2) that must be removed in later steps,[12] often lowering reaction yields. The synthesis of β-2-deoxy-D-glycosides assisted by glycosidases is reported using two mesophilic biocatalysts namely almond β-glycosidase and the *Aspergillus oryzae* β-galactosidase.[9] Long reaction times (several days), low concentrations of substrates and high concentrations of biocatalysts are typical characteristics of these reactions. Herein, we report the study of the stereochemistry of the reaction of glucal with the β-glycosidase from the archaeon *S. solfataricus*. The regioselectivity in the formation of β-2-deoxyglucosides of pyranosidic acceptors is different from that reported for mesophilic biocatalysts.

Protonation of the double bond of glucal results in the equatorially disposed proton in the product and an indication of the protonating amino acid in the active site is obtained by the use of enzyme mutated with respect to the active site nucleophile.

2. Results and discussion

The use of glucal as the donor was first studied using simple alcohols such as methanol or *n*-butanol as acceptors. These reactions, conducted with high (13–16 equiv.) excesses of acceptor and at 0.5–0.7 M initial concentration of glucal, gave good yields (50–65%) and are rapid despite the inhibitory nature of the substrate and the harsh conditions adopted with respect to the same reaction[9] conducted with mesophilic enzymes (Table 1). On using almond β-glucosidase (2000 U/mmol of glucal) other pathways (hydration and/or cleavage of the glucoside formed) competed strongly and the yields ranged from 30 to 35% after 3 days of reaction at 0.04–0.06 mM initial concentration of glucal and 60 molar excess of MeOH or allyl alcohol (Table 1).

Table 1. Syntheses of 2-deoxy-β-glucosides (entries 1–5) using the β-glycosidase from *S. solfataricus*

Entry	Acceptor (molar excess)	Glucal[a]	Product[b] (yield %)	Enzyme units[c]
1	Methanol (13)	500	**1** (50)	59
2	*n*-Butanol (16)	680	**2** (65)	59
3	2-Pentanol (16)	680	**3** (20)	59
4	Methyl-α-D-glucopyranoside (2.2)	1130	**4**+**5** (15)	59
5	Phenyl-β-D-thioglucopyranoside (1.5)	641	**6** (20)	52
6[d]	Methanol (60)	0.04	**1** (30)	2000

[a] Glucal mM concentration in the reaction mixture.
[b] The yield of the reaction is expressed with respect to glucal.
[c] The units of enzyme used per mmol of glucal (1 unit of the enzyme correspond to the amount of the enzyme which liberates 1 μmol glucose/min (salicin as substrate).
[d] Almond β-glucosidase reaction (see Ref. 9).

High concentrations of glucal (0.5–0.7 M) even at lower (13–16) molar excess of acceptors led to a doubling of the reaction yields and sensible shortening of the reaction time using our thermophilic enzyme. NMR spectroscopic study of the products indicated the formation (100%) of the β-bond and the remaining signals in the spectra are in accord with the proposed structures. In particular, in the ^{1}H NMR spectra in CD_3OD the signals of H_{eq} and H_{ax} of the C(2) methylene of methyl β-D-2-deoxyglucose (entry 1) were observed at 2.25 and 1.58 ppm respectively, the corresponding ^{13}C NMR signal being centered at 40.2 ppm. The same reaction conducted with the mutant enzyme lacking of the nucleophile in the active site (E387G) failed even in the presence of a biomimicking reactivator (sodium formate) able to restore the hydrolytic and synthetic activities of the mutant.[4]

Secondary hydroxyl group functionalization (entry 3) was less efficient and not diastereoselective with our thermophilic biocatalyst; in this reaction the yield observed was ca. 20%. In the ^{1}H NMR spectra of the compound two doublets of equal intensity, centered at 1.27 and 1.36 ppm, were observed indicating a 1:1 ratio of the two diastereomeric deoxyglucosides of 2-pentanol. The lack of diastereoselectivity and the yields observed in this case are in agreement with previous results.[2,3]

Transfer of the glucal moiety to pyranosidic substrates (entries 4 and 5, Table 1) is interesting for the formation of 2-deoxygluco disaccharides which are common structural units in many biological significant substances.[8,11] It is important to operate at low molar excess of the acceptor when useful synthetic intermediates are used in this role.

In the case of α-methyl glucoside the reaction is still operative at acceptor molar excess of 2, and at high concentration of acceptor (2.5 M) and donor (1.1 M). After 5 h all of the glucal was converted yielding 15% of disaccharide compounds. Interglycosidic linkages were determined by ^{1}H NMR spectroscopy (COSY and ^{1}H–^{13}C correlation) of acetylated derivatives. Two compounds, **4** and **5** (Fig. 2) are formed in which the deoxyglucose moiety is unexpectedly β-linked to the secondary hydroxyl groups at the O(2) and O(3) positions of α-methyl glucoside. The products were identified by 2D-NMR spectroscopy on acetylated derivatives. Following in the COSY spectrum the ^{1}H–^{1}H correlations it is possible to assign the chemical shift of the position where 2-deoxyglucose is located. ^{1}H–^{13}C correlation permits the assignment of the ^{13}C NMR chemical shifts to each position and the calculation of the glycosylation shift (ca. 5 ppm) in the carbon spectra for position 2 and 3 in compounds **5** and **4**, respectively. Similar downfield shifts were also observed for protons indicating non-acetylated (glycosylated) positions.

In the reaction using phenyl β-thioglucoside, the acceptor was used at 1.5 molar excess obtaining a 20% yield of the 2-deoxy disaccharidic derivative of thiophenol **6** (Fig. 2), a compound that is a useful starting material for oligosaccharide synthesis.[12,13] The long reaction time observed in this case (96 h) and the presence of glucose and 2-deoxyglucose in the mixture at the end of the reaction indicate both the enzymatic hydrolysis of the β-bond of the acceptor and the hydration of glucal, respectively as competing pathways. Products due to multiple transfer are not observed. However partial chemical hydrolysis cannot be excluded.

The formation of 2-deoxyglucosides of α-methyl glucopyranoside (**4** and **5**, Fig. 2) in the reaction performed by our thermophilic biocatalyst is a unique feature since the most common mesophilic almond β-glucosidase was reported to be inactive with this acceptor and only the β-1-6 linkage was formed with simple α-galactosides in the transfer of galactal catalyzed by *A. oryzae* β-galactosidase.[9] The regioselectivity of Ss-β-Gly for the transfer of 2-deoxyglucose towards the primary hydroxyl group of phenyl β-thioglucopyranoside is the same as that always observed for the transfer of glucose towards pyranosidic β-acceptors.[2] The differences observed when α-methyl glucopyranoside is the acceptor (**4** and **5**, Fig. 2), are probably due to the different position of anomeric groups of the two

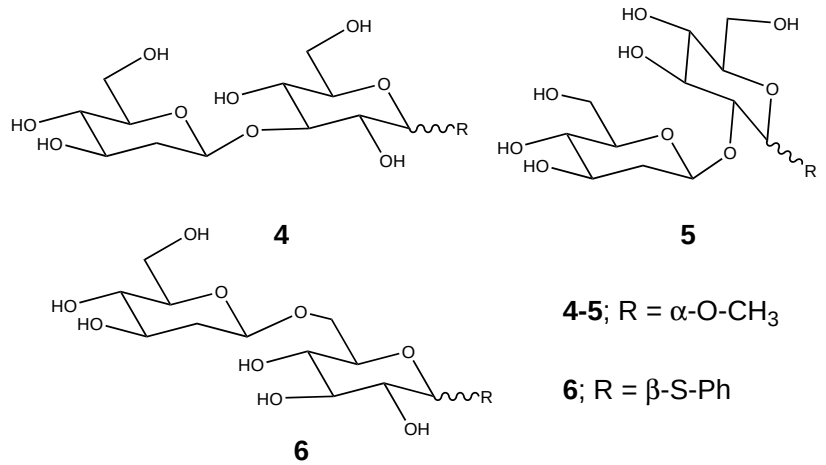

Figure 2. Pyranosidic 2-deoxy-β-D-glucosides synthesized using β-glycosidase from *S. solfataricus*.

acceptors, a structural characteristic known to be influencing the regioselectivity of glycosidases.[14] This is confirmed by the results of the reaction of wild-type enzyme with PNP-β-glucoside as donor and α-methyl glucopyranoside as the acceptor; in this case the transfer of glucose is mostly observed to the secondary (3-OH) hydroxyl group of α-methyl glucopyranoside (result not shown); however, a chromatographically inseparable mixture of minor 2-*O*-, 4-*O*- and 6-*O*-regioisomers is obtained in this reaction.

Stereochemical details of the enzymatic reaction of *S. solfataricus* β-glycosidase on glucal are interesting from a mechanistic point of view; the stereochemistry of the delivery of the proton in the active site is not known and was studied here using the wild-type enzyme for the formation of methyl and butyl 2-deoxy β-glucosides using the biocatalyst previously equilibrated in D_2O. With this experiment, in alternative to a simple enzymatic hydration of glucal, it is possible to simplify the analysis of the ^1H NMR spectra (avoiding anomeric equilibrium) and investigate the role of the hydroxylic proton in the acceptor (see below). Selective positioning of deuterium at C(2) of 2-deoxypyranose and its equatorial position were clearly established by the presence of a triplet in the ^{13}C NMR spectra shifted by 0.3 ppm with respect to the unlabelled signal (40.2 ppm) and by integration of peak areas in the ^1H NMR spectra (anomeric proton versus H_{eq} and H_{ax}), respectively. After cycles of freeze drying and redissolution of the biocatalyst in D_2O and ultrafiltration of enzyme solution, an initial 15% decrease in the integral of H_{eq} was evident indicating that it is substituted by deuterium atom; the percentage deuteration reached the maximum value of 30% after extensive deuteration process. The result obtained is evidence for proton delivery to the glucal double bond resulting in the equatorial disposition of hydrogen in the product, as observed in other retaining β-glycosidases.[6] For the first time with respect to this topic, the circumstantial negative evidence obtained with the mutant enzyme could be an indication of the identity of the protonating carboxylic group (E387).

Action of cellulases[15] on glucal shows evidence that glycosidases have catalytic groups that are functionally flexible beyond the needs of the principle of microscopic reversibility and, hence, the potential to act upon different substrates by different mechanisms.

Our observation of the limit (30%) in percentage deuteration of product even when using methanol-d_4 as the acceptor in deuterated buffer solution, indicate that, according to the general mechanism accepted for glycosyl hydrolases (see Fig. 1 for glucal, X = D), protonation of the protonating amino acid in the active site is not conceivable via direct interaction with the acid–base catalyst, a residue very closely disposed to the nucleophilic carboxylate E387; the latter would in fact be deuterated after each catalytic cycle using methanol-d_4 and direct protonation by the acid–base catalyst will increase the percentage deuteration, with respect to the ROH experiment. The definition of the precise roles of single amino acids in the active site during the enzymatic glucal transformation will be obtained by careful kinetic analysis of the 2-deoxyglucoside formation using different mutants to single amino acids in the active site.

The different regioselectivity of our enzyme with respect to mesophilic counterparts, although not necessarily linked directly to the thermophilic nature of the biocatalyst, is evidence of the importance of biodiversity in this field and is of interest for the construction of libraries of different glycosidases with different specificity.

3. Experimental

NMR spectra were recorded on a Bruker AMX 500 (500.13 MHz for ^1H and 125.75 MHz for ^{13}C) spectrometer. Samples for NMR analysis were prepared by dissolving the compounds in $CDCl_3$ or CD_3OD using the signal of the solvent as reference. Mass spectra were performed by using high-performance liquid chromatography–atmospheric-pressure chemical-ionization mass spectrometry (HPLC–APCI-MS) using a Shimadzu HPLC apparatus (LC-10ADvp) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. The temperature of the APCI source was 400°C; the HPLC column was a Phenomenex (5 μm, 150×4.5 mm) reverse phase (RP) column eluted with an isocratic step of methanol/water/acetic acid (85:15:0.1, by vol.) at a flow rate of 1 mL/min.

Commercially available donors and acceptors are obtained from Aldrich. Phenyl β-thioglucoside was obtained as described[16] by reacting α-D-glucose pentaacetate with phenylthiotrimethyl silane (60% yield). The product was characterized by ^1H and ^{13}C NMR spectroscopy as fully acetylated derivative. Glucose signals in the ^1H NMR spectra are found at δ: 4.70 (H1), 4.96 (H2), 5.20 (H3), 5.03 (H4), 3.71 (H5), 4.20 (H6). Glucose ^{13}C signals are found at δ: 85.6, 76.6, 75.7, 73.8, 69.8, 62.0.

3.1. Enzyme preparations

Wild-type (4 mg/mL, 416 U/mL)[1] and E387G mutant (1.5 mg/mL)[4] Ssβ-gly were obtained as previously reported. For the study of the stereochemistry of proton delivery wild-type enzyme was freeze dried, redissolved in D_2O and left at 70°C for 30 min (five times). The enzyme solution was then ultrafiltered (Ultrafree-CL Millipore system 30.000) and redissolved in deuterated sodium acetate buffer (three times).

3.2. Syntheses of alkyl 2-deoxyglucosides and stereochemical studies

Glucal was dissolved in the appropriate amount of the alcohol. In the methanol reaction the necessary amount of buffer was added. The enzyme solution was added and the reactions started at 70°C under agitation in a

sealed vial up to total glucal consumption, ca. 16 h, as monitored by TLC (EtOAc/MeOH/H_20, 72:5:4, by vol. or EtOAc/MeOH 72:5, v/v). Products were purified by standard chromatographic procedures and identified by ^1H and ^{13}C NMR spectroscopy. Methanol, methanol-d_4 and *n*-butanol acceptors were used for stereochemical studies and for reaction with mutant enzyme.

3.2.1. β-Methyl-2-deoxyglucoside. ^1H NMR: δ 4.59, 3.91–3.16, 2.09 (H_{eq}), 1.59, 1.47 (H_{ax}), 1.36, 0.92; ^{13}C NMR: δ 102.2, 77.9, 72.9, 72.4, 62.9, 56.81, 40.2.

3.2.2. β-*n*-Butyl-2-deoxyglucoside. ^1H NMR: δ 4.52, 4.02–3.28, 2.25 (H_{eq}), 1.58 (H_{ax}); ^{13}C NMR: δ 101.1, 77.9, 73.0, 72.5, 69.9, 62.9, 40.4, 32.8, 20.2, 14.2.

3.2.3. β-2-Pentyl-2-deoxyglucoside. ^1H NMR: δ 4.80, 4.10–3.35, 2.20 (H_{eq}), 1.72–1.40, 1.36, 1.27, 1.07; ^{13}C NMR: δ 100.8, 98.4, 77.9, 77.0, 74.3, 73.2, 73.0, 72.6, 72.5, 63.0, 62.9, 40.9, 40.7, 40.6, 39.9, 22.0, 19.6, 14.5.

3.3. Syntheses of pyranosidic 2-deoxyglucosides

α-Methyl glucoside (1.5 mmol) and glucal (0.68 mmol) were dissolved in phosphate buffer (400 μL, 50 mM pH 6.5). Wild-type enzyme preparation (200 μL, 0.8 mg total protein) were added and the reaction started at 70°C under agitation in a sealed vial for 5 h up to total consumption of glucal as monitored by TLC (EtOAc/MeOH/H_2O, 70:20:10, by vol.). Chromatographic prepurification on a RP-8 glass column eluting with water furnished two disaccharidic compounds which were then peracetylated (Ac_2O/pyridine, overnight room temperature) and purified by preparative TLC (*n*-hexane/EtOAc, 6:4, v/v). Total yield of the two deoxyglucosides of α-methyl glucoside is 15% with respect to glucal (8.5% for **5** and 6.4% for **4**, Fig. 2). Compound **5**: ^1H (^{13}C) NMR signals δ, Glucose: 4.92 (99.6) H1, 3.72 (78.2) H2, 5.36 (71.4) H3, 4.99 (69.1) H4, 3.95 (67.1) H5, 4.05–4.25 (62.5) H6; 2-deoxyglucose: 4.62 (101.2) H1, 1.76–2.19 (36.2) H2, 4.95 (69.3) H3, 4.96 (68.2) H4, 3.58 (72.4) H5, 4.25 (62.2) H6. $[\alpha]_D^{20}$ +36.7 (*c* 3.4, CH_2Cl_2). Compound **4**: ^1H (^{13}C) NMR signals δ, Glucose: 4.92 (96.8) H1, 4.79 (73.0) H2, 4.09 (76.4) H3, 4.98 (68.5) H4, 3.93 (67.4) H5, 4.03–4.12 (61.8) H6; 2-deoxyglucose: 4.63 (99.9) H1, 1.65–2.18 (36.2) H2, 4.93 (70.4) H3, 4.91 (68.2) H4, 3.52 (71.7) H5, 4.32–4.02 (61.9) H6. $[\alpha]_D^{20}$ +37.8 (*c* 2.6, CH_2Cl_2). APCI mass spectra of both regioisomers shows base peak at 615 $(M+Na)^+$.

Thiophenyl β-D-glucopyranoside (968 mg, 3.5 mmol) and glucal (337 mg, 2.3 mmol) dissolved in phosphate buffer (3 mL, 50 mM pH 6.5) were added to wild-type enzyme (400 μL) and the reaction started at 70°C under agitation in a sealed vial. After 24 h a second aliquot (200 μL) of enzyme is added and reaction left up to total consumption of glucal (96 h, total reaction time). Chromatographic prepurification on a RP-8, acetylation and purification by silica give total yield of 20% with respect to glucal of compound **6** (Fig. 2). Compound **6**: ^1H (^{13}C) NMR signals δ, Glucose: 4.77 (85.8) H1, 4.96 (69.7) H2, 5.23 (73.7) H3, 4.93 (68.7) H4, 3.76 (77.4) H5, 3.87–3.62 (68.2) H6; 2-deoxyglucose: 4.57 (100.1) H1, 1.71–2.25 (36.5) H2, 4.97 (70.1) H3, 4.92 (68.8) H4, 3.57 (71.7) H5, 4.27–4.06 (62.3) H6. $[\alpha]_D^{20}$ −24.0 (*c* 15, CH_2Cl_2). APCI mass spectra shows base peak at 693 $(M+Na)^+$.

Acknowledgements

S. Zambardino and V. Mirra (ICMB-NMR service) are acknowledged for technical assistance. The present research was partially supported by the Italian PNRA project.

References

1. Moracci, M.; Ciaramella, M.; Rossi, M. *Methods Enzymol*. **2001**, *330*, 201–215.
2. Trincone, A.; Improta, R.; Nucci, R.; Rossi, M.; Gambacorta, A. *Biocatalysis* **1995**, *12*, 77–88.
3. Huneke, F. U.; Bailey, D.; Nucci, R.; Cowan, D. *Biocatal. Biotrans*. **2000**, *18*, 291–299.
4. Moracci, M.; Trincone, A.; Perugino, G.; Ciaramella, M.; Rossi, M. *Biochemistry* **1998**, *37*, 1726–17270.
5. Trincone, A.; Perugino, G.; Rossi, M.; Moracci, M. *Bioorg. Med. Chem Lett*. **2000**, *10*, 365–368.
6. Sinnot, M. L. *Chem. Rev*. **1990**, *90*, 1171–1202.
7. Lai, E. C. K.; Morris, S. A.; Street, I. P.; Withers, S. G. *Bioorg. Med. Chem*. **1996**, *4*, 1929–1937.
8. Wentworth, D. F.; Wolfenden, R. *Biochemistry* **1974**, *23*, 4715–4720.
9. Petit, J. M.; Paquet, F.; Beau, J. M. *Tetrahedron Lett*. **1991**, *32*, 6125–6128.
10. Legler, G.; Roeser, K.; Illig, H. K. *Eur. J. Biochem*. **1979**, *101*, 85–92.
11. Weiser, W.; Lehman, J.; Matsui, H.; Brewer, C. F.; Henre, E. J. *Arch. Biochem. Biophys*. **1992**, *292*, 493–498.
12. Marzabaldi, C. H.; Franck, R. W. *Tetrahedron* **2000**, 8385–8417.
13. Nicolau, K. C.; Seitz, S. P.; Papahatjis, D. P. *J. Am. Chem. Soc*. **1983**, *105*, 2431–2434.
14. Nilsson, K. G. *Carbohydr. Res*. **1987**, *15*, 95–103.
15. Kanda, T.; Brewer, C.; Okada, G.; Hehre, E. J. *Biochemistry* **1986**, *25*, 1159–1165.
16. Buskas, T.; Garegg, P. J.; Konradsson, P.; Maloisel, J. L. *Tetrahedron: Asymmetry* **1994**, *5*, 2187–2194.