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EFFICIENT INHIBITION OF MUSCLE AND LIVER GLYCOGEN PHOSPHORYLASES BY A NEW GLUCOPYRANOSYLIDENE-SPIRO-THIOHYDANTOIN

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Abstract: Reaction of *C*-(1-bromo-1-deoxy-β-D-glucopyranosyl)formamide **2** with thiocyanate ions was the key step of a short synthesis of D-glucopyranosylidene-spiro-thiohydantoin **7** which proved to be a potent inhibitor of muscle and liver glycogen phosphorylases.

Non-insulin dependent *diabetes mellitus* (NIDDM or Type II diabetes) can be controlled mainly by dietary regulation and with the use of hypoglycaemic agents.[1] Blood glucose concentration, however, cannot be regulated very efficiently by these methods. Therefore, considerable efforts have been made to understand the action and elicit inhibition, using glucose derivatives, of glycogen phosphorylase (GP) the main regulatory enzyme of blood sugar level.[2-6] The most potent inhibitor, known to date, of muscle GP*b* is a glucopyranosylidene-spiro-hydantoin derivative[7] **5** ($K_i = 3.1 \pm 0.2$ μM), for which several multistep syntheses have been published.[2,3,7] Apart from the lengthiness of these synthetic sequences, unfavourable stereoselectivity as well as anomerizations in the intermediates strongly decrease the stereochemical efficiency of this compound's production. It is therefore unlikely that these syntheses will be useful for the preparation of the large amounts of this inhibitor[3] required for more elaborate biological investigations. Obviously, any readily available potent inhibitor could facilitate deeper understanding of the mechanism of action of GP and validate the concept of its inhibition as potential therapy of diabetes.

Herein we report the synthesis of a glucopyranosylidene-spiro-thiohydantoin (**7**) based on a six step, simple, highly chemo-, regio-, and stereoselective procedure starting from D-glucose and the kinetic data demonstrating efficient

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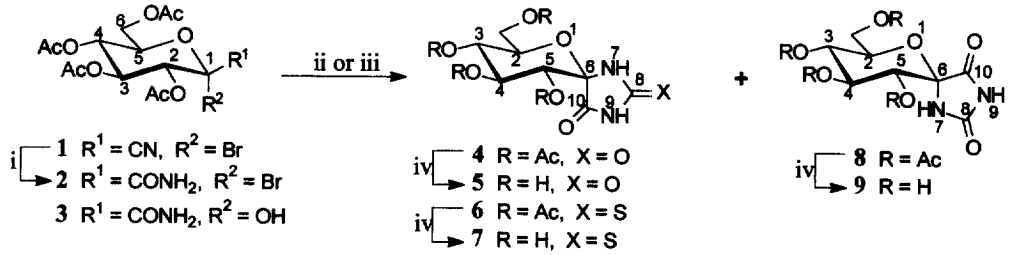
inhibition of muscle and liver glycogen phosphorylases by this compound.

Chemistry

1-Bromo-1-deoxy-β-D-glucopyranosyl cyanide **1**,[8] readily available by radical-mediated bromination[9] of the corresponding acetylated β-D-glucopyranosyl cyanide,[10] was transformed into the *C*-(1-bromo-1-deoxy-β-D-glucopyranosyl)formamide **2**[11] by $TiCl_4$ mediated partial hydrolysis.[12,13] Ring closure of **2** was effected by AgOCN in dry nitromethane whereby formation of a mixture of spiro-hydantoins **4**[11] and **8**[11] (ratio 1 : 10 by ^1H NMR) was observed. Transformation of **2** with AgSCN or KSCN in dry nitromethane gave **6**[11] as a single anomer. In each reaction a small amount of **3**[11] could be isolated as an unavoidable by-product. Deprotection of **4**, **6**, and **8** under Zemplén-conditions afforded **5**,[7] **7**,[11] and **9**,[7] respectively.



Scheme: i $TiCl_4$, H_2O, AcOH, 0 °C–r. t.; ii AgOCN (4 eq) dry CH_3NO_2, 80 °C; iii AgSCN (4 eq) or KSCN (2 eq), dry CH_3NO_2, 80 °C; iv NaOMe, MeOH, r.t.

Constitution of the spiro-hydantoins was established using NMR methods.[11,14] Compounds **4-9** showed characteristic ^1H and ^{13}C resonances expected for the (thio)hydantoin rings[14-16] and corresponding long-range $^{13}C/^1H$ correlations were identified in the HMBC spectra. The pyranose ring conformations were deduced from $^3J_{HH}$ values as $^4C_1$ for each derivative and the C-6 configurations determined as *S* for **6** and **7** and *R* for **8** on the basis of $^3J_{H\text{-}5,C\text{-}10}$ values.[11] The ^1H and ^{13}C NMR spectra for **5** and **9** were identical with the reported ones.[7] The configurational assignments were supported by ^1H chemical shift rules recently established[13] for spiro-hydantoins.

Kinetic Studies

Glycogen phosphorylase *b* was isolated from rabbit skeletal muscle according to the method of Fischer and Krebs[21] with the difference of using 2-mercaptoethanol instead of L-cysteine. Phosphorylase kinase was prepared from rabbit skeletal muscle[22] for converting muscle phosphorylase *b* into phosphorylase *a* as described in.[23] Hepatic phosphorylase

b and *a* were prepared from rat livers according to the reported method.[24] For the preparation of liver phosphorylase *a* glucagon (100 μg/kg) was injected intravenously 30 sec before cutting the jugular veins and 50 mM NaF was added to the buffers to keep the enzyme in the phosphorylated form in all subsequent steps. Liver phosphorylase *b* was prepared from non-glucagonized rats without NaF addition to the buffers. Phosphorylase activities were assayed in the direction of glycogen synthesis at 30°C with 10 μg/ml enzyme, 1% glycogen, in the absence of AMP (muscle or liver phosphorylase *a*) or in the presence of AMP (1 mM AMP for muscle and 2 mM AMP for liver phosphorylase *b*) with or without hydantoins **5**, **7**, and **9** in 50 mM triethanolamine/HCl (pH 6.8) buffer, 100 mM KCl, 1 mM dithiothreitol and 1 mM EDTA. The kinetic studies were performed as described previously[25] with the exception that higher D-glucose 1-phosphate concentrations were used for the assay of liver phosphorylases as suggested by Stalmans and Hers.[26] Inhibition of liver phosphorylase *b* by compound **7** according to Lineweaver-Burk is shown in Fig. 1. The inhibitor constant for **7** calculated from a Dixon plot (Fig. 2) is $K_i = 7$ μM.

Figure 1

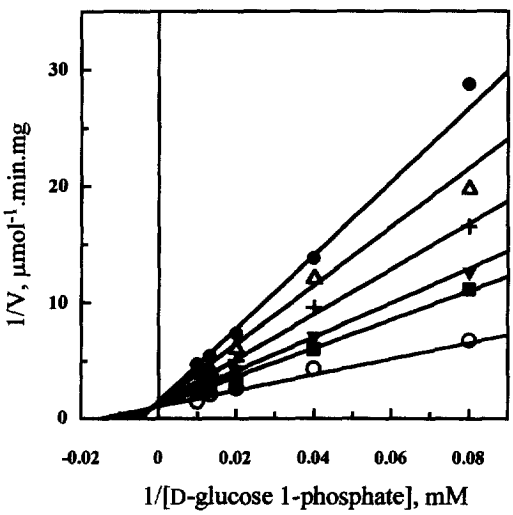


Figure 2

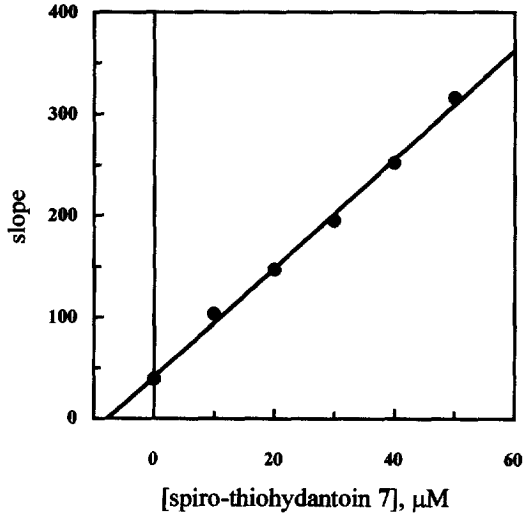


Figure 1. Double-reciprocal plot for liver phosphorylase *b* at constant concentrations of AMP (1 mM) and glycogen (1%), and varying concentrations of D-glucose 1-phosphate. Concentrations of **7** were as follows: 0 (○), 10 (■), 20 (▼), 30 (+), 40 (Δ) and 50 μM (●). The plots were analysed by a non-linear data-analysis program.[27]

Figure 2. Determination of K_i value by replotting the slopes in Figure 1 against the inhibitor concentrations.[28] The means of standard errors for all calculated kinetic parameters averaged to less than 10%.

Inhibitor constants (K_i) of thiohydantoin **7** for muscle and liver phosphorylases are summarized in Table 1. For comparison, inhibitor constants of hydantions **5** and **9** determined with muscle and liver phosphorylases are also presented together with those published previously[2,7] for muscle phosphorylase *b*. Compound **5** proved to be a potent inhibitor as described before,[7] the epimeric **9** was, however, found to be more effective than reported.[2] Liver phosphorylase *a* was only slightly inhibited by **9**. It can be concluded that phosphorylase *a* activities (assays in the absence of AMP) are less sensitive to the inhibitory action of compounds **5** or **7**.

Table 1.
Inhibitor constans (K_i [μM]) of spiro-(thio)hydantoins **5**, **7**, and **9** for muscle and liver phosphorylases

Type of phosphorylase	**5**	**7**	**9**
Liver phosphorylase *b*	12.8 ± 1.1	7.0 ± 1.0	n. d.*
Liver phosphorylase *a*	16.5 ± 1.3	29.8 ± 2.9	2050 ± 180
Muscle phosphorylase *b*	4.2 ± 0.4	5.1 ± 0.5	105 ± 9
	(3.1 ± 0.2[7])		(320[2])
Muscle phosphorylase *a*	26.0 ± 2.4	10.9 ± 1.0	n. d.*

*not determined

Conclusion

The newly synthesized spiro-thiohydantoin derivative of D-glucose (**7**) was found to be a very potent inhibitor of muscle and liver glycogen phosphorylases *b* and *a*. The inhibitory efficiency of **7** is comparable to that of the best substance (**5**) known for this purpose to date. Due to the minor difference in the structures of **5** and **7** the important hydrogen bond between N7-H and His377, detected by X-ray crystallography for muscle GP*b*–**5** complex,[29] may also exist in the enzyme–**7** complexes resulting in similar inhibitions. Purified liver phosphorylases were used for the first time in our experiments implying a potential role of inhibitors in the hepatic glycogen metabolism.

Acknowledgements

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11. Characterization of the new compounds (isolated yields are given):
    2 yield: 68 %; mp: 119-121°C, $[\alpha]_D$ +124 (c 1.09, $CHCl_3$), ^{1}H-NMR ($CDCl_3$) δ 6.50 (1H, s, NH_2), 6.02 (1H, s, NH_2), 5.44 (1H, t, J = 9.4 Hz, H-3), 5.20 (1H, d, J = 9.4 Hz, H-2), 5.16 (1H, t, J = 9.4 Hz, H-4), 4.33-4.22 (3H, m, H-5,6,6'); ^{13}C-NMR ($CDCl_3$) δ 166.9 ($CONH_2$, $^3J_{H\text{-}2,CO(NH2)}$ = 1.5 Hz), 92.7 (C-1), 74.3, 71.6, 69.6, 66.7 (C-2 to C-5), 60.7 (C-6); Anal.: calcd. for $C_{15}H_{20}NO_{10}Br$ (454.24): C 39.6, H 4.43, N 3.08, Br 17.59; found: C 38.74, H 4.74, N 3.21, Br 17.17.
    3 yield: 19 %; mp: 175-177°C; $[\alpha]_D$ +35 (c 1.35, acetone); ^{1}H-NMR (CD_3OD) δ 5.48 (1H, dd, J = 9.9, 9.5 Hz, H-3), 5.29 (1H, d, J = 9.9 Hz, H-2), 5.15 (1H, dd, J = 10.1, 9.5 Hz, H-4), 4.32 (1H, dd, J = 12.2, 5.2 Hz, H-6), 4.25 (1H, ddd, J = 10.1, 5.2, 2.3 Hz, H-5), 4.16 (1H, dd, J = 12.2, 2.3 Hz, H-6'); ^{13}C-NMR (CD_3OD) δ 172.5 ($CONH_2$, $^3J_{H\text{-}2,CO(NH2)}$ ~ 1 Hz), 95.6 (C-1), 72.7, 72.5, 70.6, 70.1 (C-2 to C-5), 63.7 (C-6); High resolution MS: calcd. for $C_{15}H_{22}NO_{11}$ $[M+H]^+$ 392.11926, found 392.11933, Δ (mmu) 0.06.
    4 yield: 4 %; oil; $[\alpha]_D$ +18 (c 1.01, $CHCl_3$); ^{1}H-NMR ($CDCl_3$): δ 8.14 (1H, s, H-9), 6.23 (1H, s, H-7), 5.92 (1H, quasi t, J = 10.1, 9.8 Hz, H-4), 5.32 (1H, d, J = 10.1 Hz, H-5), 5.2 (1H, quasi t, J = 10.2, 9.8 Hz, H-3), 4.7 (1H, ddd, J = 10.2, 4.1, 2.1 Hz, H-2), 4.29 (1H, dd, J = 12.7, 4.1 Hz, CH_2), 4.13 (1H, dd, J = 12.7, 2.1 Hz, CH_2); ^{13}C-NMR ($CDCl_3$): δ 168.9 (C-10), 154.6 (C-8), 86.4 (C-6), 71.5, 70.6, 70.1, 67.8 (C-2 to C-5), 61.8 (CH_2); High resolution MS: calcd. for $C_{16}H_{21}N_2O_{11}$ $[M+H]^+$ 417.1139, found 417.1143.
    6 yield 57 %; mp 223-224°C; $[\alpha]_D$ +2 (c 2.12, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 8.38 (1H, s, H-9), 7.21 (1H, s, H-7), 5.92 (1H, dd, J = 10.1, 9.8 Hz, H-4), 5.34 (1H, d, J = 10.1 Hz, H-5), 5.21 (1H, quasi t, J = 9.9, 9.8 Hz, H-3), 4.74 (1H, ddd, J = 9.9, 4.2, 2.0 Hz, H-2), 4.29 (1H, dd, J = 12.7, 4.2 Hz, CH_2), 4.14 (1H, dd, J = 12.7, 2.0 Hz, CH_2); ^{13}C-NMR ($CDCl_3$) δ 182.1 (C-8), 171.0 (C-10, $^3J_{H\text{-}5,C\text{-}10}$ = 6.1 Hz), 86.8 (C-6), 70.7, 69.8, 69.7, 67.5 (C-2 to C-5), 61.6 (CH_2); ^{15}N-NMR ($CDCl_3$, referenced to NH_4Cl as external standard) δ 130.9 (N-7), 160.5 (N-9); High resolution MS: calcd. for $C_{16}H_{21}N_2O_{10}S$ $[M+H]^+$ 433.09167, found 433.09172, Δ (mmu) 0.11.
    7 yield: 78 %; amorphous product; $[\alpha]_D$ +19 (c 2.34, CH_3OH); ^{1}H-NMR (CD_3OD) δ 4.23 (1H, ddd, J = 10.0, 5.4, 2.2 Hz, H-2), 4.13 (1H, t, J = 9.6 Hz, H-4), 3.83 (1H, dd, J = 12.2, 2.2 Hz, CH_2), 3.68 (1H, dd, J = 12.2, 5.4 Hz, CH_2) 3.54 (1H, d, J = 9.6 Hz, H-5), 3.37 (1H, dd, J = 10.0, 9.6 Hz, H-3); ^{13}C-NMR (CD_3OD) δ 185.9 (C-8), 174.6 (C-10, $^3J_{H\text{-}5,C\text{-}10}$ = 6.3 Hz), 90.7 (C-6), 76.8, 74.4, 74.1, 70.9 (C-2 to C-5), 62.7 (CH_2); High resolution MS: calcd. for $C_8H_{13}N_2O_6S$ $[M+H]^+$ 265.04941, found 265.04953, Δ (mmu) 0.16.
    8 yield: 25 %, amorphous product; $[\alpha]_D$ +29 (c 2.54, $CHCl_3$); ^{1}H-NMR ($CDCl_3$): δ 8.98 (1H, s, H-9), 8.3 (1H, s, H-7), 5.6 (1H, d, J = 10.2 Hz, H-5), 5.56 (1H, dd, J = 10.2, 9.0 Hz, H-4), 5.26 (1H, dd, J = 10.2,

9.0 Hz, H-3), 4.28 (1H, dd, J = 12.6, 4.4 Hz, CH_2), 4.19 (1H, dd, J = 12.6, 2.1 Hz, CH_2), 4.03 (1H, ddd, J = 10.2, 4.4, 2.1 Hz, H-2); ^{13}C-NMR ($CDCl_3$): δ 167.9 (C-10, $^3J_{H\text{-}5,C\text{-}10}$ = 2.8 Hz), 156.8 (C-8), 87.4 (C-6), 71.8, 71.6, 68.2, 68.0 (C-2 to C-5), 61.9 (CH_2). High resolution MS: calcd. for $C_{16}H_{21}N_2O_{11}$ $[M+H]^+$ 417.1145, found 417.1146, Δ (mmu) 0.13.

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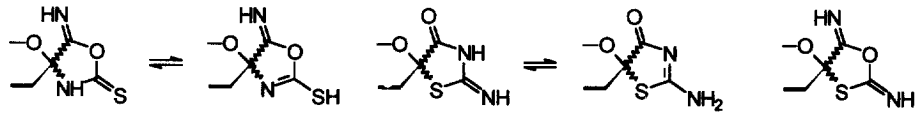
14. NMR data allowed to rule out possible other isomers which could have, theoretically, arisen from nucleophilic attack by the sulfur rather than the nitrogen atom of the thiocyanate ion (**B**, **C**) or ring closure by attack of the amide oxygen instead of nitrogen (**A**, **C**). The measured ^{13}C chemical shifts are characteristic[15,16] of C=S (182.1 ppm in **6** and 185.9 ppm in **7**) and C=O groups (171.0 ppm in **6** and 174.6 ppm in **7**) and ^{15}N shifts (δN 130.9 ppm and 160.5 ppm in **6**) correspond to ring NHs in hydantoins.[17] In contrast, C=N bonds present in each of the alternative structures **A**-**C** would require δC and δN values to be in the ranges between 152-166 ppm[18] and 200-300 ppm,[19] respectively. The only glycosylidene-spiro-thiohydantoin derivatives which we could locate in the literature are analogues[20] of (+)-hydantocidin characterised by ^{1}H NMR, IR and MS but not by ^{13}C and ^{15}N NMR data.



A **B** **C**

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