Microbiological *N*-Deoxygenation and *C*-Oxygenation of Pioglitazone-*N*-oxide in a Single Fermentation

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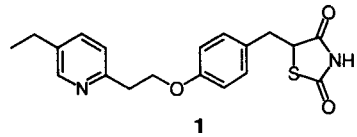
Oxygenation of pioglitazone-*N*-oxide by a microorganism isolated from soil was accompanied by *N*-deoxygenation to produce the pioglitazone metabolites 5-[4-[2-[5-(1-hydroxyethyl)-2-pyridyl]ethoxy]benzyl]-2,4-thiazolidinedione and 5-[4-[2-(5-acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione. The oxygenating/deoxygenating organism has been characterized as *Streptomyces hygroscopicus* strain 02179 (UC 11099). The culture has been deposited with Agricultural Research Service, USDA, with accession number NRRL 18975.

The molecule pioglitazone[1] (**1**; 5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione) is one of several members of a large family of substituted benzylthiazolidinediones which have been extensively studied for their properties as hypoglycemic agents.[2,3] Many members of this family, including pioglitazone, have their origin in the laboratories of the Takeda Chemical Industries.[4] One particularly interesting aspect of this class of potential drugs is that their mechanism of action differs from that of the classical sulfonylureas, which are thought to act by promoting insulin release.[2] The benzylthiazolidinediones, by contrast, appear to act by increasing the sensitivity of various cells and tissue to existing levels of insulin.[2] Following extensive evaluation[5,6] of an earlier analog, ciglitazone,[7] as a potential drug, recent efforts have been aimed at the second generation molecule, pioglitazone. Among the preclinical and clinical work carried out with pioglitazone has been the study of the metabolism of the compound in several animal species[8,9] and in humans.[9] The two most abundant metabolites in humans are compounds **2** and **3**, resulting from the oxygenation of the ethyl side chain attached to the pyridine ring. Of further interest is the fact that when both of these metabolites were tested in various assays, they elicited hypoglycemic responses which were similar in nature and potency to those of pioglitazone itself.[9] Further pharmacological evaluation of these metabolites therefore required a convenient method for their preparation.

As an alternate to the total synthesis of metabolites **2** and **3**,[9] we turned to the possibility of using microbiological oxygenation as a means for obtaining these compounds. The use of microorganisms to oxygenate foreign organic molecules at positions difficult to access by other methods now has a lengthy history in which literally thousands of compounds have been functionalized by this technique.[10~15] Among the applications for which the method has been used is the synthesis of metabolites of drug molecules. In this report, we describe the results of our efforts to prepare metabolites **2** and **3** by microbial oxygenation.

Discussion

We decided to use both pioglitazone (**1**) and pioglitazone-*N*-oxide (**4**) as potential substrates for the desired oxygenation reactions. There were two reasons for this choice. First, pioglitazone is notorious for its



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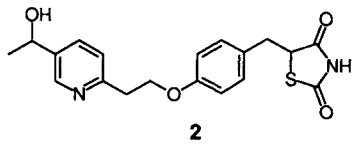

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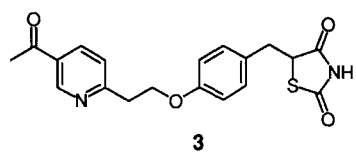

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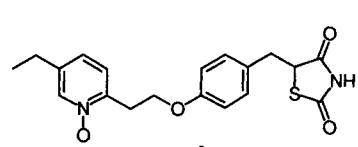

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physical properties. Presumably because of its zwitterionic character, the molecule is relatively insoluble in many organic solvents as well as in water and we were concerned that this insolubility might prevent oxygenation of the molecule. This characteristic is somewhat alleviated in the *N*-oxide since now a zwitterion no longer exists. Second, heterocyclic amines have been reported to undergo biological *N*-oxidations, so by using the *N*-oxide as a potential substrate, complications arising from formation of the *N*-oxide during the fermentation would be avoided.

It was convenient for us to use a random screening approach to discover an organism capable of oxygenating **1** and/or **4**. Therefore, screening was begun by adding 20 mg of either **1** or **4** dissolved in DMF (0.2~0.3 ml) to each vigorously growing fermentation of 100 ml total volume. Fermentations were continued 1~3 days after addition of the substrate, then CH_2Cl_2 was added to the fermentation flask and, taking advantage of the mechanical shakers used for the fermentations, the contents of the flasks were extracted by continuing the shaking action for 1~2 hours. After filtration, separation, and concentration of the organic extract, the extract was examined by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

Among the first forty-five cultures screened and extracted was one (identified as isolate 02179 for screening purposes; the taxonomy of the culture is discussed below) for which TLC results suggested the formation of one or more oxygenation products from **4**. When this extract was examined by HPLC, very little remaining **4** was detected. Further, several peaks for new components were seen and when compared to authentic samples of the metabolites one of these new peaks co-eluted with the alcohol **2** and a second new peak co-eluted with ketone **3**. Clearly, not only had the desired oxygenation apparently occurred in this fermentation but deoxygenation of the pyridine-*N*-oxide had accompanied the oxygenation process. Another small peak in the HPLC chromatogram was observed to have the same retention time as pioglitazone and in later experiments was proven to be pioglitazone. From this result it is clear that *N*-deoxygenation can occur without being accompanied by oxygenation of the ethyl side chain. In a separate experiment, we also found that pioglitazone (**1**) underwent partial oxygenation to **2** and **3** when placed in a fermentation with isolate 02179 under the same conditions used for the oxygenation of **4**.

A time-course and substrate level study was carried out in shake flasks for the conversion of **4** by isolate 02179. Flasks of the culture containing 20, 40 or 60 mg of **4** were shaken for 24, 48, or 72 hours after addition of the substrate and then were worked up and analyzed as described above. These experiments clearly showed that good conversion of substrate to products was achieved at the highest substrate level (60 mg per flask) within 48 hours after addition to the culture. An additional set of experiments was done using substrate levels of 90 and 120 mg per flask over a time course of 48, 72, or 96 hours. At these higher substrate levels, conversion to products was still good but larger amounts of pioglitazone were present in the extracts. Consequently, subsequent studies were done using 50~60 mg of **4** per 100 ml of fermentation.

Our preliminary efforts to carry out the fermentation of **4** in 10-liter tanks have given variable results. Consumption of substrate in these tank fermentations appears to be slower than in shake flasks and there also is more pioglitazone produced which complicates the purification of products **2** and **3**.

Fermentations equivalent to 10- or 12-liter batches were done in shake flasks, the contents of the flasks were pooled at the end of the fermentation, and the pooled mixture was extensively extracted with CH_2Cl_2. Our best results, described in detail in the Experimental Section, were obtained from such a 12 liter pool starting with a total of 6.0 grams of **4**. Following chromatographic separation and purification using several chromatographic columns, pure recrystallized alcohol **2** (1.35 g, 22%) and pure recrystallized ketone **3** (0.684 g, 11%) were obtained. The structures of **2** and **3** isolated from the fermentation process were confirmed by comparison of spectral properties with those of authentic samples. Some enantioselectivity is seen in the oxygenation process as shown by the observation of an optical rotation for **2**, but the stereochemical purity of the product has not been determined. These results do offer an effective alternative to total synthesis as a method for preparation of pioglitazone metabolites **2** and **3**.

As noted above, at the outset of this work we were concerned about the possible oxidation of the pyridine in pioglitazone to a pyridine-*N*-oxide, a transformation for which there is precedent.[12] We therefore used the preformed pyridine-*N*-oxide **4** as a substrate and observed the opposite result, *i.e.*, deoxygenation of the *N*-oxide, a biotransformation for which there also is literature precedent.[12] The observation of efficient reductive and oxidative transformations of a substrate in a single fermentation is unusual although there is no reason why it should be unexpected. The question as to whether any of the oxygen atoms from the *N*-oxide are transferred into oxygenation products **2** and **3** is intriguing but has not been examined. Amine *N*-oxides are capable of serving as oxygen atom donors in some metalloporphyrin-catalyzed oxygen transfers to hydrocarbons.[16] Metalloporphyrin systems have been extensively studied as a mimics for cytochrome P-450 catalyzed oxygenations[17] which we assume is the mode of oxygenation observed here for pioglitazone.

Taxonomic studies of isolate 02179 have been carried out and the details are described in the Experimental Section. The organism grows well, producing a white aerial mass which becomes hygroscopic and turns dark gray to black on some media. The mature spore chains are long and form tight spirals. The spore surface is rugose (see Fig. 1). The organism has a wide range of

growth temperature and utilizes most carbon sources. Based on the morphological and cultural characteristics and the whole-cell hydrolysate analysis, the organism was determined to be a strain of *Streptomyces hygroscopicus* now designated as strain 02179. The organism has been assigned Upjohn Company Culture Collection number UC 11099 and has been deposited with Agricultural Research Service, USDA, under accession number NRRL 18975.

Once the organism was characterized as a strain of *S. hygroscopicus*, forty-two other strains of this microorganism which were available to us through the Upjohn Culture Collection were screened for their ability to transform pioglitazone-*N*-oxide into metabolites **2** and **3**. Metabolites **2** and/or **3** were detected by HPLC analysis in fermentations of fourteen of these *S. hygroscopicus* strains with the most abundant quantities produced by cultures UC 5176, UC 5208, UC 8625, UC 8657, and UC 8675. However, none of these were as effective as UC 11099 in the production of the desired metabolites.

Experimental

Taxonomy

Source of Strain

UC 11099 was isolated from a soil sample collected in Tennessee.

Morphology[18]

UC 11099 produced long spore chains of tight spirals. Spore surfaces were rugose (Fig. 1). Vegetative hyphae showed no evidence of fragmentation. Motile spores and sporangium-like bodies were not observed.

Cultural Characteristics[19,20]

On most ISP media, UC 11099 grew well and produced a gray aerial mass which became black and hygroscopic as the incubation time was prolonged beyond two weeks. The reverse side of the colony was yellowish brown to yellowish gray. Melanoid pigment was not produced, nor was any soluble pigment (Table 1). The culture strongly

Fig. 1. Scanning electron micrograph of UC 11099, showing spiral sporophores with rugose surfaced spores.

Bar represents 1 μm.

Table 1. Cultural characteristics of UC 11099.[a]

Medium	Parameter[b]	Chip[c]	Color[c]	Comments
ISP medium 2	G			Good
(Yeast extract - malt extract agar)	AM	263~266	White→dark gray	
	R	77	Medium yellow brown	
	SP	—		
ISP medium 3	G			Good
(Oatmeal agar)	AM	263~266	White→dark gray	
	R	93	Yellow gray	
	SP	—		
ISP medium 4	G			Good
(Inorganic salts - starch agar)	AM	263~266	White→dark gray	Starch
	R	77	Medium yellow brown	Hydrolyzed
	SP	—		
ISP medium 5	G			Good
(Glycerol - asparagine agar)	AM	92	Yellow white	
	R	93	Light yellow brown	
	SP	—		
ISP medium 6	G			Good
(Peptone - yeast extract agar)	AM	93	Yellow gray	
	R	76	Yellow white	
	SP	—		
ISP medium 7	G			Fair
(Tyrosine agar)	AM	93	Yellow gray	
	R	76	Yellow white	
	SP	—		
ISP medium 8				Nitrate not
(Nitrate broth)				reduced

[a] All readings were taken after 14 days at 28°C.

[b] G: Growth, AM: aerial mass, R: reverse, SP: soluble pigment.

[c] Color was compared with chip standards from the ISCC-NBS (Inter-society Color Council-National Bureau of Standards) color name chart.

solubilized tyrosine and weakly solubilized casein. It did not solubilize calcium malate or xanthine. It hydrolyzed starch on ISP medium 4. Nitrate was not reduced to nitrite.

Physiological Characteristics[19]

UC 11099 utilized a wide range of sugars, including glucose, arabinose, xylose, mannitol, fructose, rhamnose, and raffinose. It did not utilize sucrose or cellulose. Utilization of inositol was questionable (Table 2). The culture grew in the temperature range of 24~45°C with an optimum range of 28~37°C. It did not grow at 55°C.

Cell-wall and Sugar Analysis[21]

The cell wall of UC 11099 contained LL-diaminopimelic acid; no *meso* isomer was detected. The whole-cell hydrolysate contained galactose.

Taxonomic Position

The morphology, cultural and physiological characteristics, and the whole-cell hydrolysate analysis of the culture revealed that UC 11099 is a species of *Streptomyces*. The hygroscopic colony appearance and the rugose surfaced spores further place the culture in the species *hygroscopicus*. Thus, UC 11099 was identified as *S. hygroscopicus*. The number UC 11099 refers to the designation given the culture within the Upjohn Culture Collection. It has been deposited with Agricultural Research Service, USDA, under accession number NRRL 18975.

Table 2. Utilization of carbon sources by UC 11099.

Carbon source[a]	Utilization[b]	Comments
D-Glucose (positive control)	++	Good growth, aerial mass white turning to dark gray
L-Arabinose	++	Good growth, aerial mass white turning to dark gray
Sucrose	−	No growth
D-Xylose	++	Good growth, aerial mass white turning black
i-Inositol	±	Scant black aerial mass
D-Mannitol	++	Good growth, aerial mass white turning black
D-Fructose	++	Good growth, aerial mass white turning black
Rhamnose	+	Fair growth, some black aerial mass
Raffinose	+	Fair growth, some black aerial mass
Cellulose	−	Very scant growth
No carbon (negative control)	−	Very scant growth

[a] Carbon sources were added to ISP medium 9 to give a final concentration of 1%.

[b] ++ = Growth on tested carbon is similar to or greater than growth on positive control.
+ = Growth is somewhat less than positive control.
± = Growth is somewhat more than negative control but much less than positive control.
− = Growth is similar to that on negative control, or no growth.

Biotransformation Process

Frozen agar plugs of *S. hygroscopicus* (UC 11099, NRRL 18975, stored over liquid N_2) were aseptically transferred into a 100 ml volume of GS-7 medium (sterile) contained in a 500-ml large mouth shake flask. The GS-7 medium was composed of 25 g/liter each of Cerelose and Pharmamedia in tap water. The pH of the medium was adjusted to 7.2 with ammonium hydroxide. The medium was sterilized by autoclaving for 30 minutes. The inoculated GS-7 medium was shaken at 250 rpm at 28°C for 72 hours. This fermentation was used to inoculate additional flasks (500 ml volume) containing 100 ml of sterilized medium in the amount of 5% by volume. These additional flasks are shaken at 250 rpm and 28°C for 48 hours. At this time the substrate (pioglitazone or pioglitazone-*N*-oxide) dissolved in *N*,*N*-dimethylformamide (DMF) was added to the fermentation in a range between 20 and 60 mg/flask using no more than 0.3 ml of DMF per flask. The flasks were shaken an additional 24~96 hours.

5-[4-[2-[5-Ethyl-1-oxo-pyrid-2-yl]ethoxy]benzyl]-2,4-thiazolidinedione (**4**, Pioglitazone-*N*-oxide)[22]

m-Chloroperbenzoic acid (67% purity, 42.5 g, 0.165 mole of peroxide) was added in three portions over a period of 45 minutes to a stirred mixture of **1** (52.57 g, 0.15 mole) in $CHCl_3$ (1300 ml) and CH_3OH (375 ml) at room temperature. A completely clear solution was seen within 30 minutes after the addition was finished. The solution was stirred at room temperature for 24 hours. TLC (10% CH_3OH in $CHCl_3$) showed a trace of material having the same Rf as **1**, but did not change upon addition of additional *m*-chloroperbenzoic acid (4.8 g) and an additional 4 hours of stirring. The reaction solution was stirred with sat'd aq $NaHCO_3$ (600 ml) for 30 minutes, the mixture was transferred to a separatory funnel with an H_2O rinse, and the layers separated. The organic layer was washed with H_2O (500 ml), with aq Na_2SO_3 (10 g in 200 ml H_2O), with H_2O (500 ml), and was dried over Na_2SO_4. The solution was filtered, concentrated on the rotary evaporator, and the oily residue was dissolved immediately in CH_3CN (350 ml). A white solid slowly crystallized and was collected by filtration, giving **4** (45.03 g) as a crystalline, colorless solid. One recrystallization from CH_3CN gave 41.97 g (0.112 mole, 75%) of **4**, mp 160~162.5°C.

Anal Calcd for $C_{19}H_{20}N_2O_4S$:
C 61.28, H 5.41, N 7.52, S 8.61.
Found:
C 60.82, H 5.69, N 7.40, S 7,45.

Isolation of 5-[4-[2-[5-(1-Hydroxyethyl)-2-pyridyl]-ethoxy]benzyl]-2,4-thiazolidinedione (**2**) and 5-[4-[2-(5-Acetyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione (**3**)

A solution of pioglitazone-*N*-oxide (**4**, 6.0 g, 0.0161 mole) in DMF (total volume of solvent and substrate, 24 ml) was divided equally among 120 flasks each containing 100 ml of medium and growing culture and shaking at 28°C was continued for 24 hours. The contents of the flasks were pooled in a 20-liter glass carboy, CH_2Cl_2 (6 liters) was added, and the two-phase system was stirred vigorously with an air driven motor for 2 hours. The solvent layers separate slowly and after about 24 hours, the CH_2Cl_2 layer is siphoned off under a slight vacuum. The extraction process was repeated by adding fresh CH_2Cl_2 in the amount removed to the pooled fermentation, stirring vigorously, and removing CH_2Cl_2 by siphon. This extraction process was done a total of ten times. Extractions 1 ~ 7 (total volume 18.2 liters) were combined and extraction 8 ~ 10 (total volume 12.2 liters) were combined.

Combined extracts 1 ~ 7 were dried (Na_2SO_4), filtered, and concentrated. The residual material was chromatographed over silica gel (40 ~ 63 μm, 400 g, preconditioned with *i*-propyl alcohol - EtOAc - hexane - glacial acetic acid (5 : 65 : 29.5 : 0.5)) packed in a 4.7 × 45 cm Michel-Miller column. The residue was applied to the column by first dissolving in 1 : 1 $CHCl_3$ - CH_3OH, mixing with gravity grade silica gel (35 g), and removing the solvent on the rotary evaporator under reduced pressure. The dry solid was placed in a second 4.7 × 45 cm Michel-Miller column held in a vertical position. The empty space in the column was filled with 40 ~ 63 μm silica gel and the column was eluted with the same solvent mixture used to precondition the column. Fractions of 50 ml volume were collected and were pooled based on the results of tlc analysis.

Fractions 21 ~ 33 were pooled and contained ketone **3** and a small amount of pioglitazone (total, 1.20 g). This material was chromatographed over 200 g of 40 ~ 63 μm silica gel using EtOAc to apply the material to the column and *i*-propyl alcohol - EtOAc - hexane - gl. acetic acid (5 : 65 : 29.5 : 0.5) to elute the column. Fractions of 30 ml volume were collected with fractions 11 ~ 15 containing the desired ketone (0.955 g) still mixed with pioglitazone. The mixture was again chromatographed over 200 g of 40 ~ 63 μm silica gel, eluting with $CHCl_3$ - *i*-propyl alcohol - acetic acid (94 : 5 : 1) and collecting 40 ml fractions. Fractions 12 ~ 15 contained crystalline ketone **3** (0.602 g) and fractions 19 ~ 26 contained impure pioglitazone (0.184 g). The combined fractions 12 ~ 15 were recrystallized from acetone-hexane, giving **3** (0.511 g), mp. 113 ~ 115°C. The ^1H NMR spectrum in $CDCl_3$ of the sample is identical to the spectrum of an authentic sample of ketone **3**.

Fractions 34 ~ 40 were pooled and contained a mixture of ketone **3** and alcohol **2** (total, 0.41 g). This mixture was plated onto 8 g of silica gel and placed in line in front of a column of 200 g of 40 ~ 63 μm silica gel. The system was eluted with $CHCl_3$ - *i*-propyl alcohol - acetic acid (94 : 5 : 1) and fractions of 45 ml volume were collected. Fractions 15 ~ 20 contained pure ketone **3** [0.082 g, total (0.602 g + 0.082 g) 0.684 g, 0.00183 mole, 11%). Fractions 21 ~ 23 contained impure ketone **3** (0.030 g). Fractions 191 ~ 196 contained pure alcohol **2** (0.151 g).

Fractions 41 ~ 63 were pooled and contained primarily alcohol **2** as a yellow solid (1.34 g). The solid was dissolved in methanol-EtOAc and while hot was decolorized with activated charcoal. After filtration and removal of solvent, an off-white solid (1.26 g) remained and was chromatographed over 200 g of 40 ~ 63 μm silica gel. Fractions of 50 ml volume were collected and fractions 85 ~ 106 contained pure crystalline alcohol **2** (1.0 g). Recrystallization from acetone-hexane gave **2** (0.884 g), mp 154.5 ~ 155.5°C; $[\alpha]_D$ −8.1° (*c*, 0.620, EtOH).

Fractions 64 ~ 77 were pooled and contained a mixture of alcohol **2** and pioglitazone-*N*-oxide (total, 0.11 g).

Fractions 78 ~ 120 were pooled and contained primarily pioglitazone-*N*-oxide (0.282 g). The material was plated onto silica gel (7 g) and placed in line in front of a column of 200 g of 40 ~ 63 μm silica gel. The system was eluted with $CHCl_3$ - *i*-propyl alcohol - acetic acid (94 : 6 : 1) (2 liters) followed by $CHCl_3$ - *i*-propyl alcohol - acetic acid (88 : 10 : 2). Pure pioglitazone-*N*-oxide (0.186 g, 3%) was eluted in fractions 33 ~ 42 (45 ml volume).

Combined extracts 8 ~ 10 were dried (Na_2SO_4), filtered, and concentrated. The residue was plated onto silica gel (25 g, 40 ~ 63 μm), which was placed ahead of a column of 200 g of 40 ~ 63 μm silica gel and eluted with *i*-propyl alcohol - EtOAc - hexane - acetic acid (5 : 65 : 29.5 : 0.5). Fractions (45 ml volume) 29 ~ 40 contained impure alcohol **2** (0.295 g), which was rechomatographed over 200 g of 40 ~ 63 μm silica gel using 5 liters of $CHCl_3$ - *i*-propyl alcohol - acetic acid (94 : 5 : 1) followed by a 88 : 10 : 2 mixture of the same. Fractions (45 ml volume) 156 ~ 176 contained pure alcohol **2** [0.201 g, total (0.151 g + 1.00 g + 0.201 g) 1.35 g, 0.00363 mole, 22%]; $[\alpha]_D$ −6.7° (*c*, 0.780, EtOH). The ^1H NMR spectrum of the alcohol is identical with that of an authentic sample of **2**.

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