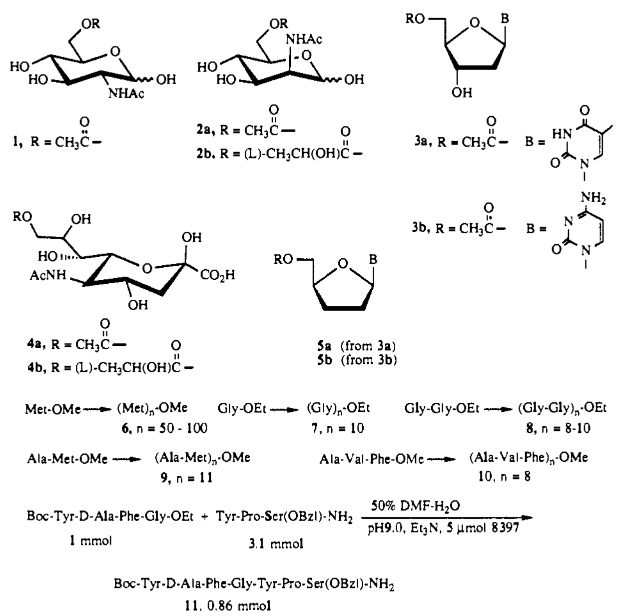
**Table I.** Kinetic Constants for Subtilisin BPN' and Mutants[a]

| | BPN' | | | 8350 | | | 8397 | | | 8399 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| substrate | $k_{cat}$, $s^{-1}$ | $K_m$, $\mu M$ | $k_{cat}/K_m$, $M^{-1} s^{-1}$ | $k_{cat}$, $s^{-1}$ | $K_m$, $\mu M$ | $k_{cat}/K_m$, $M^{-1} s^{-1}$ | $k_{cat}$, $s^{-1}$ | $K_m$, $\mu M$ | $k_{cat}/K_m$, $M^{-1} s^{-1}$ | $k_{cat}$, $s^{-1}$ | $K_m$, $\mu M$ | $k_{cat}/K_m$, $M^{-1} s^{-1}$ |
| Suc-AAPF-pNA[b] | 47 | 172 | $2.7 \times 10^5$ | 130 | 160 | $8.1 \times 10^5$ | 74 | 97 | $7.6 \times 10^5$ | 76 | 112 | $6.8 \times 10^5$ |
| NTCI[c] | 0.2 | 76 | $2.2 \times 10^3$ | 0.6 | 67 | $9.6 \times 10^3$ | 0.3 | 42 | $7.1 \times 10^3$ | 0.2 | 33 | $6.0 \times 10^3$ |
| Z-Lys-SBzl | 46 | 531 | $8.7 \times 10^4$ | 33 | 536 | $6.1 \times 10^4$ | 70 | 900 | $7.8 \times 10^4$ | 32 | 948 | $3.4 \times 10^4$ |
| Bz-Tyr-OEt | 70 | 1700 | $4.1 \times 10^4$ | 233 | 818 | $2.9 \times 10^5$ | 82 | 2386 | $3.4 \times 10^5$ | 73 | 2358 | $3.1 \times 10^5$ |

[a] Conditions are the same as those described previously.[4] [b] *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitrophenylanilide. [c] *N*-*trans*-Cinnamoylimidazole.

specificity for the L-amino acid at the $P_2$ position. The relative rates for the hydrolysis of L vs D diastereomers at an ester group are approximately >100:1.

To evaluate the synthetic utility of the mutant enzyme 8397, several regioselective reactions were conducted in DMF. Compounds **1**, **2a**, **3a**, and **3b** were prepared in 90–95% yield by reaction of the corresponding free sugars or nucleosides with 10 equiv of vinyl acetate in DMF. Compound **2b** was prepared in




50% yield with >98% regioselectivity by reaction of the corresponding free sugar with ethyl L-lactate in the presence of 10% water. Compounds **2a** and **2b** were further converted to **4a** and **4b**, respectively, via reaction with pyruvate catalyzed by sialic acid aldolase. Compounds **3a** and **3b** were deoxygenated via a radical reaction to the corresponding 2,3-dideoxy nucleosides **5a** and **5b**. The enzyme was also used in the enantioselective hydrolysis of synthetic racemic amino acid esters including *N*-(ethoxycarbonyl)furylglycine and *N*-acetylhomophenylalanine methyl esters, and the results are the same as those obtained with 8350, 8399, and the wild-type enzymes. At 50% conversion in each case, both product and the unreacted substrate were recovered in >98% ee. Application of 8397 to peptide synthesis in 50% DMF, pH 9, was also conducted,[4] and similar results were obtained for the wild-type and the three mutant enzymes, except that 8397 is about 10 times more efficient and 8350 and 8399 are about 5 times more efficient than the wild-type enzyme, presumably due to the improved stability of the mutant enzyme. Polymerization of single amino acid, dipeptide, and tripeptide methyl esters to compounds **6–10** and segment condensation for the synthesis of **11** were conducted under the same conditions.[10]

(10) The condensation product was purified and characterized to be identical with that prepared previously.[4] The polymers were purified by gel filtration chromatography, and the degree of polymerization was estimated on the basis of the molecular weight and the relative intensity of the C-terminal-$OCH_3$ compared to the integrated α-H's as determined by $^1H$ NMR. (Bibbs, J. A.; Zhong, Z.; Wong, C.-H. In *Materials Synthesis Utilizing Biological Processes*; Ricke, P. C., Calvert, P. D., Alper, M., Eds.; Materials Research Society: Pittsburgh, PA, 1990; p 223.) Short polypeptides were obtained mainly due to their low solubilities in the solvent system.

In summary, the technique of site-directed mutagenesis has proven useful for the improvement of enzyme stability in polar organic solvent. The dramatic increase in the stability of subtilisin 8397 in DMF makes it a useful enzyme for the transformation of various organic compounds which require DMF as solvent. Further study along this line should provide rich experimental data useful for engineering enzymes to be used in organic media.[11]

**Supplementary Material Available:** Procedures for syntheses and experimental data for **1–10** (7 pages). Ordering information is given on any current masthead page.

(11) For a review in the field, see: Arnold, F. H. *TIBTECH* **1990**, *8*, 244. Hwang, J.; Arnold, F. H. *Biocatalysis*; Blanch, H. W., Clark, D. S., Eds.; In press.

## Epoxyquinones from 2,5-Dihydroxyacetanilide: Opposite Facial Specificity in the Epoxidation by Enzymes from *Streptomyces* LL-C10037 and *Streptomyces* MPP 3051

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*Received September 24, 1990*

We have previously reported the detailed biosynthesis[1,2] of the antitumor metabolite LL-C10037α (**1**),[3] produced by *Streptomyces* LL-C10037,[4] from the shikimate pathway via 3-hydroxyanthranilic acid (**2**). Six steps from **2** to **1** were identified by whole-cell and cell-free studies,[2] with the latter implicating the acetamidoquinone **3** as the epoxidation substrate to yield epoxyquinone **4**.[5] Antibiotic MM14201 (**5**),[6] produced by *Streptomyces* MPP 3051, is the desacetyl enantiomer of **1**[3] and could be derived either by a pathway totally different from the biosynthesis of **1** or, more simply, by the same pathway (exclusive of stereochemistry) with the addition of a deacetylation as the last step. We now report that the correct epoxidase substrate is the acetamidohydroquinone **6** and that partially purified extracts from the two organisms epoxidize **6** to yield the enantiomeric products **4** and **7**, respectively.

Initially a mixture of **3** and either NADH or NADPH was treated with a cell-free extract of *S.* LL-C10037; the choice of substrate and cofactor was based on the in vivo incorporation of **3**[2] and the reported enzymatic epoxidation of nanaomycin A.[7]

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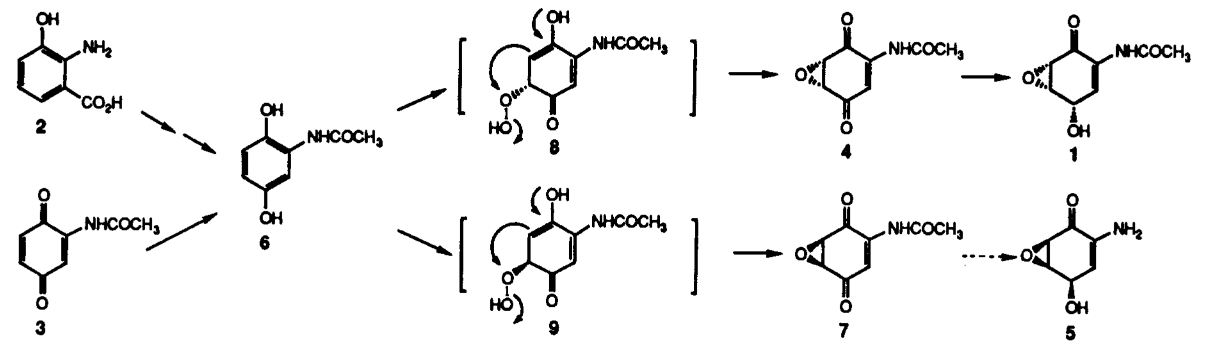
0002-7863/91/1513-684$02.50/0 

Scheme I



However, during subsequent purification of the *S.* LL-C10037 epoxidase to apparent homogeneity,[8] it was discovered that **6** is the true substrate and is efficiently epoxidized[9] in the absence of any added cofactor.[10]

Concurrent with this, according to our standard protocol, washed mycelia (2.7 g) from a 300-mL fermentation of *S.* MPP 3051 were disrupted by sonication[11] and partially purified by sequential treatment with protamine sulfate[12] and ammonium sulfate (AS).[13] The 62–92% AS pellet was redissolved in 12.0 mL of 50 mM potassium phosphate, pH 7.0, containing 20% glycerol and 0.2 mM EDTA, and distributed equally to 12 reaction tubes (final volume 2.0 mL, 125 mM potassium phosphate, pH 7.0, 1.0 mM substrates[14]). After 10-min incubation at 30 °C, the combined reaction mixtures were saturated with solid NaCl and extracted with EtOAc. Concentration to dryness and chromatography of the mixture on silica gel 60, eluting with $CHCl_3$/MeOH/hexane = 5/1/1, afforded crude product that was recrystallized from MeOH/EtOAc to give 1.3 mg (30%) of crystalline **7**, whose $^1H$ NMR spectrum was identical with that of authentic **4**.

The epoxyquinone **4** from the *S.* LL-C10037 pathway[5] has been shown to have the absolute stereochemistry indicated[3] and has a specific rotation of $[\alpha]^{20}{}_D$ +115.6° (*c* 0.5, MeOH).[15] The epoxyquinone **7** obtained with the *S.* MPP 3051 enzyme has a specific rotation of $[\alpha]^{20}{}_D$ −112.2° (*c* 0.1, MeOH)[16] and is, therefore, the enantiomer of **4** (see Scheme I).

Numerous epoxyquinones have been characterized; often the related quinones and/or epoxyquinols occur as their cometabolites. In all cases studied, the epoxide oxygen has been derived from molecular oxygen.[1,7,17,19] Both hydroquinone and quinone have been proposed as the epoxidation substrate,[1,17,20–23] but in only two previous cases has such an enzyme activity been isolated. A crude cell-free extract of *Streptomyces rosa* var. *notoensis* catalyzed the epoxidation of a naphthoquinone (nanaomycin A) only in the presence of NADH or NADPH,[7] while a cell-free particulate preparation of *Penicillium patulum* catalyzed the epoxidation of gentisyl alcohol (a hydroquinone) in the absence of any added cofactor,[24] as we now report for two *Streptomyces* enzymes. Consistent with the mechanism proposed for the *P. patulum* enzyme, reaction of **6** with enzyme-activated oxygen would yield the intermediate **8** or **9**, which would then decompose to **4** or **7**, respectively.

Enantiomeric natural products, ostensibly derived from the same substrate, have only rarely been found, and most of these occur in the terpenoid area; in a few cases the responsible complementary enzymes have been isolated.[25–32] The two complementary epoxidases reported herein may each be viewed as a paradigm for hydroquinone epoxidation, presumably leading to the epoxyquinones found in many metabolic pathways. Studies on the reaction mechanism(s) as well as determination of the three-dimensional features of the active sites that control the opposite facial specificities of these two *Streptomyces* enzymes will be the subject of future communications.

**Acknowledgment.** Dr. Donald Borders, Lederle Laboratories, Pearl River, NY, is thanked for a strain of *S.* LL-C10037 and for a sample of LL-C10037α. Dr. Stephen Box, Beecham Pharmaceuticals, Betchworth, Surrey, United Kingdom, is thanked for a strain of *S.* MPP 3051 and for a sample of MM 14201. Dr. James Soodsma is thanked for helpful discussions as this work

(8) The purification of the epoxidases will be reported elsewhere.

(9) Assays by HPLC on a Waters Assoc. $C_{18}$ Radialpak column using 15% aqueous $CH_3CN$ + 0.1% TFA, 1.0 mL/min, detection at 225 nm. In 5-min incubations, >50% conversion of **6** to **4** could be obtained, while only with cell-free extract (CFE) could **4** be obtained from **3** (11.6%) without added cofactor,[2] apparently due to endogeneous NADH.

(10) Cell-free activity capable of reducing **3** to **4** in the presence of NADH has been obtained and partially purified. **3** can be reduced chemically with either NADH or NADPH.

(11) The washed cells were suspended in 50 mM potassium phosphate, pH 7.0, containing 20% glycerol, 0.2 mM EDTA, 1.0 mM phenylmethanesulfonyl fluoride, and 3.0 mg/mL polyvinylpolypyrrolidone. The mixture was sonicated (4 °C, maximum power, 90% duty cycle, pulsed for 4 × 15 s) and centrifuged (4 °C, 23200*g*, 10 min) to yield the cell-free extract (CFE).

(12) The CFE was brought to 0.01% protamine sulfate by addition of a 2.0% solution, stirred at 4 °C for 0.5 h, and centrifuged (4 °C, 38400*g*, 20 min); the supernatant was then treated with solid AS.

(13) We have purified this enzyme to near homogeneity,[8] and the correct substrate is **6**.

(14) Prior to recognizing the correct substrate, **3** and NADH were used for enzyme incubations.

(15) **4** was obtained by PCC oxidation of pure authentic **1**.[3]

(16) **7** had been previously reported with $[\alpha]^{20}{}_D$ = −99° (*c* 0.5, MeOH) from PCC oxidation of MT 35214, the product from acetylation of MM 14201.[6]

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evolved. Support from National Science Foundation Grant CHE-8711102 (S.J.G.) and from a Faculty Research Award from the American Cyanamid Co. (S.J.G.) is greatly appreciated. The multinuclear Bruker AC 300 NMR spectrometer was purchased through grants from the Public Health Service Division of Research Resources (RR04039-01) and the National Science Foundation (CHE-8712343) to Oregon State University.

**Note Added in Proof.** A third example of hydroquinone epoxidation is the dihydro-vitamin K epoxidase activity associated with "vitamin K-dependent carboxylase".[33,34]

## Polyacrylamides Bearing Pendant $\alpha$-Sialoside Groups Strongly Inhibit Agglutination of Erythrocytes by Influenza Virus[1]

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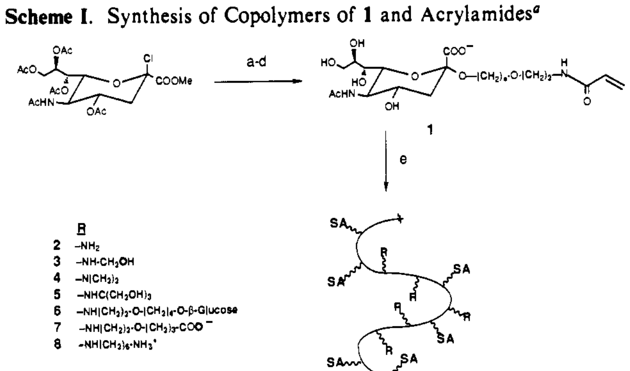
The initial step of invasion of a mammalian cell by influenza virus is the binding of the viral membrane protein hemagglutinin (HA) to sialic acid (SA) residues of cell surface glycoproteins and glycolipids.[2] Tight-binding inhibitors of this association are potential inhibitors of influenza infection. Systematic examinations of monomeric derivatives of sialic acids have not, to date, revealed compounds binding significantly more tightly to HA than $\alpha$-glycosides of sialic acid.[3,4]

Although the binding of HA to $\alpha$-sialosides is weak ($K_D \sim 2 \times 10^{-3}$ M),[3] the binding of influenza virus to cells appears to be strong. The qualitative difference between the strength of interaction between monomeric HA and methyl $\alpha$-sialoside, and that between virus and cell surface, probably reflects the polyvalency of the latter interaction.[5] This inference is supported by the observation that certain glycoproteins, especially $\alpha_2$-macroglobulins, having high contents of sialic acid are strong inhibitors of virus-induced agglutination of erythrocytes.[6,7] Of the known sialic acid containing glycoproteins, only a few are capable of protecting erythrocytes from viral agglutination, and it is difficult to pinpoint the origin of this activity. We believe that the number and accessibility of sialic acid groups in these glycoproteins play key roles.

The structures of these complex, naturally occurring, polyvalent hemagglutination inhibitors are largely unknown, and it is impractical to prepare close analogues of them by synthesis, or to study relations between their structure and strength of inhibition. We therefore sought practical routes to synthetic macromolecules to which sialic acid groups could be attached, and in which composition and strucxture could be varied readily. Here we report that such substances can be prepared conveniently by free-radical copolymerization of **1**, an acrylamide derivative of sialic acid, with acrylamide and its derivatives (Scheme I). The most active of these copolymers are powerful inhibitors of hemagglutination by influenza virus.[8]

Polymerizations followed standard procedures.[9] We have not characterized these polymers fully, but dialysis of representative

**Scheme I.** Synthesis of Copolymers of **1** and Acrylamides[a]




[a] (a) $HO(CH_2)_4O(CH_2)_3NHCbz$, Ag-salicylate, $C_6H_6$, 25 °C, 3 days; (b) 1 N NaOH, 25 °C, 12 h; (c) $H_2$/5% Pd–C, MeOH, 25 °C, 6 h; (d) *N*-(acryloyloxy)succinimide, $Et_3N$, $H_2O$, 25 °C, 12 h; (e) $CH_2{=}CHCOR$, 4,4'-azobis(4-cyanopentanoic acid), $h\nu$ (365 nm), 25 °C, 5 h.

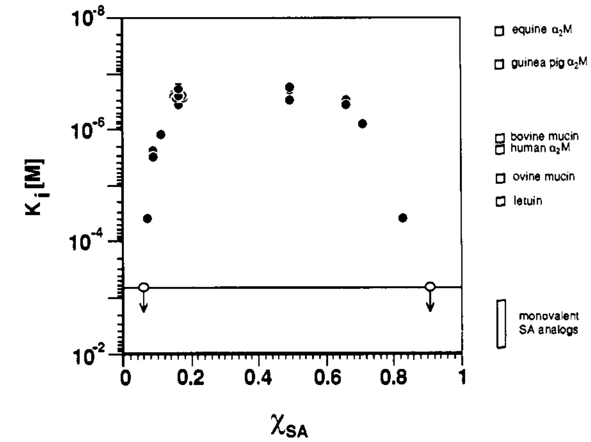



**Figure 1.** Inhibition of hemagglutination of erythrocytes by poly(**1**-*co*-acrylamide). The inhibition constant, $K_i$, is calculated on the basis of sialic acid groups in solution. $\chi_{SA}$ is the mole fraction of **1** in the mixture of **1** and acrylamide used in forming the polymer. The cluster of data at $\chi_{SA}$ = 0.17 corresponds to 10 independent experiments producing the values of $K_i = 1.8 \times 10^{-7}$ (2×), $3.0 \times 10^{-7}$ (7×), and $3.6 \times 10^{-7}$ (1×). The reference data listed at the right margin of the figure for proteins and analogues of SA are taken from refs 4 and 6. We have confirmed the values of bovine mucin and fetuin independently. Polymers having values for $K_i > 6.25 \times 10^{-4}$ M (the horizontal line in the figure) were not examined quantitatively, and the points (O) at $\chi_{SA}$ = 0.063 and 0.91 represent lower limits.

**Table I.** Values of $K_i$ for Copolymers Prepared from 1:1 Molar Mixtures of **1** and Acrylamides

| acrylamide | $K_i$, M |
|---|---|
| **2** | $3.0 \times 10^{-7}$ |
| **3** | $3.0 \times 10^{-7}$ [a] |
| **4** | $2.5 \times 10^{-6}$ |
| **5–8** | $>6 \times 10^{-4}$ |

[a] This copolymer is only partially soluble.

(1) This research is part of a collaboration with J. Skehel, J. R. Knowles, M. Karplus, and D. C. Wiley and was supported by the NIH, Grant GM 39589. A.S. is a Postdoctoral Fellow of the Swiss Academy of Sciences.

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(9) Compound **1** and acrylamide (or an analogue) were mixed in aqueous solution at pH 7.0 (with a total concentration of acrylamide moieties of 1.0 M) containing initiator (0.02 M). The solution was deoxygenated by passing argon through it, and polymerization was initiated by using a UV lamp (365 nm).

 