ulation J (τ = 538 fs) simultaneously with K (τ = 656 fs) (Scheme II). Relatively slow decay of J back to bR results in the apparent generation of a more blue-shifted intermediate (Δt = 10 ps, Figure 1g). Time resolved resonance Raman spectroscopy has the potential of detecting J and testing the validity of our model. Furthermore, the transition from J (S_1) to the ground state is potentially observable by using time resolved fluorescence spectroscopy. We predict this transition to be very broad ($\Delta\bar{\nu} \approx \sim 6000$ cm^{-1}) with a Franck-Condon maximum at $\sim 1.6\ \mu$.

The Biosynthesis of LL-C10037α from the Shikimate Pathway

Yvonne G. Whittle and Steven J. Gould*[1]

Department of Chemistry, Oregon State University Corvallis, Oregon 97331

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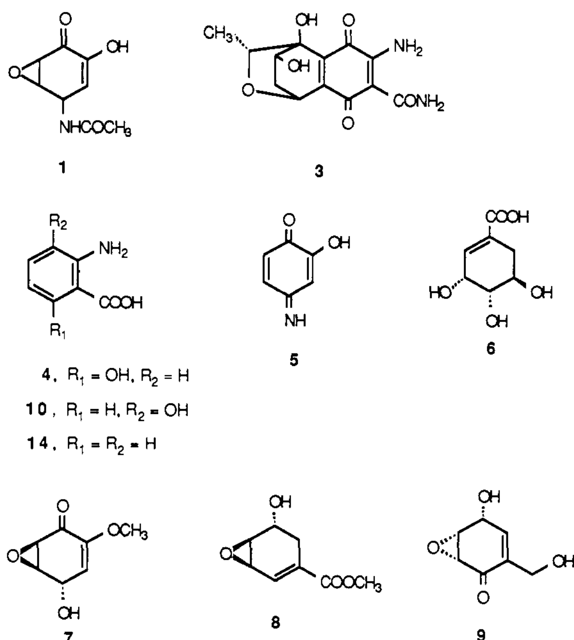
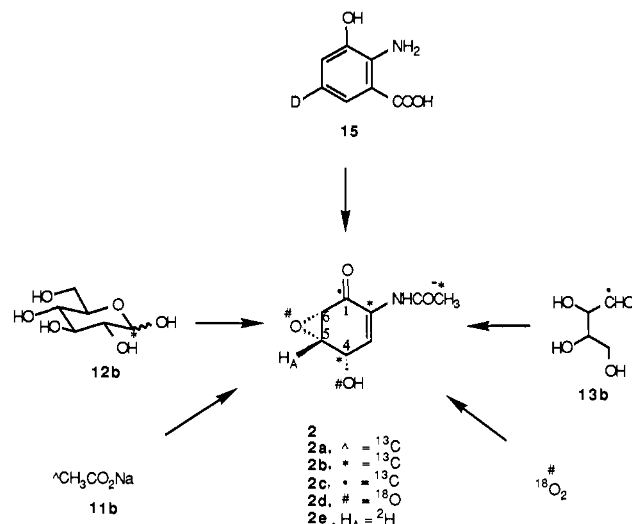
LL-C10037α, an antitumor metabolite of *Streptomyces* LL-C10037 first assigned structure **1**,[2] has recently been shown to have the corrected structure **2**.[3] Several related structures have been isolated from a wide variety of microorganisms. For example, sarubicin A, **3**, contains a quinone moiety which we have shown to be derived from the shikimate metabolite 6-hydroxyanthranilic acid, **4**.[4] In addition, the hydroxyazaquinone **5**[5] is derived from shikimic acid **6** via *p*-aminobenzoic acid (PABA),[5,6] while chaloxone **7**,[7] isolated with its cometabolite methyl anhydroshikimic acid **8**,[8] may also be shikimate derived. On the other hand, the members of a large family of metabolites[9] represented by epoxydon, **9**,[10] have been clearly shown to be acetate-derived polyketides.[9,10] We now report evidence that **2** is derived via the shikimic acid pathway with 3-hydroxyanthranilic acid, **10**, as the key intermediate.



Cultures of *S.* LL-C10037 were grown in shake flasks (200 mL of broth in a 1-L Erlenmeyer) at 28 °C as previously described.[2] After incubation for 122 h, the broth was obtained free of the mycelia, adjusted to pH 4.7 (KH_2PO_4), and saturated with $(NH_4)_2SO_4$. Extraction with ethyl acetate, concentration in vacuo, and column chromatography (20 g silica gel 60, eluted with 20% hexanes in ethyl acetate) typically gave 30 mg of crystalline LL-C10037α which was then recrystallized from methanol.

For feeding experiments cultures were usually grown for 96 h before potential precursors were added in a sterile manner through a Millipore filter unit, and the broth was worked up after a total of 122 h. A mixture of sodium [1-^{14}C]acetate (9 μCi), **11a**, sodium acetate (23.6 mg), **11**, and sodium [2-^{13}C]acetate



(68.3 mg), **11b**,[12] was fed first, and workup yielded 60 mg of **2a** (1.3% incorporation of ^{14}C). The 100.6-MHz ^{13}C NMR spectrum[13] of **2a** revealed enrichment (6.4%) only in the acetamide methyl group, revealing that the carbocycle is not polyketide in origin.

Feeding[14] a mixture of [1-^{14}C]-D-glucose, **12a**, (5.5 μCi) and [1-^{13}C]-D-glucose (0.996 g), **12b**,[15] yielded 35 mg of **2b**. The ^{13}C NMR spectrum of enriched **2b** showed labeling at C-2 (5.94%) and C-4 (7.02%), clearly indicative of a shikimate-type pathway;[16] the acetamide methyl was also enriched (4.69%), due to the incorporation of glycolysis-derived acetylCoA. The ring labeling pattern is inconsistent with the intermediacy of PABA (C-2 and C-6 would have been labeled in this case).[17]

To determine the correct orientation of the apparent shikimic

(1) Career Development Awardee of the National Cancer Institute (CA00880), 1979-1984.
(2) Lee, M. D.; Fantini, A. A.; Morton, G. O.; James, J. C.; Borders, D. B.; Testa, R. T. *J. Antibiot.* **1984**, *37*, 1149.
(3) Whittle, Y. G.; Keszler, D. A.; Gould, S. J., unpublished results of X-ray diffraction study. The absolute configuration shown in based on a circular dichroism study.
(4) Hillis, L. R.; Gould, S. J. *J. Am. Chem. Soc.* **1985**, *107*, 4593.
(5) Mize, P. D.; Jeffs, P. W.; Boekelheide, K. *J. Org. Chem.* **1980**, *45*, 3543.
(6) Tsuji, H.; Bando, N.; Ogawa, T.; Sasaoka, K. *Biochim. Biophys. Acta* **1981**, *667*, 326.
(7) Fex, T.; Trofast, J.; Wickberg, B. *Acta Chem. Scand., Ser. B* **1981**, *35*, 91.
(8) Fex, T.; Wickberg, B. *Acta Chem. Scand., Ser. B* **1981**, *35*, 97.
(9) Berdy, J. *Handbook of Antibiotic Compounds*; CRC Press Inc., 1980; Vol. 3 pp 343-366. Turner, W. B.; Aldridge, D. C. *Fungal Megabolites II*; Academic Press: New York, 1983; pp 62-67.
(10) Nabeta, K.; Ichihara, A.; Sakamura, S. *Agr. Biol. Chem.* **1975**, *39*, 409.
(11) Read, G.; Westlake, D. W. S.; Vining, L. D. *Can. J. Chem.* **1969**, *47*, 1071.
(12) [2-^{13}C]Sodium acetate (90 atom % C-13) was obtained from MSD Isotopes.
(13) Spectrum taken in 0.4 mL MeOH-d_4 with a Bruker AM 400 spectrometer at 100.6 MHz (sweep width = 2500 Hz, 64K data points, pulse width = 32.5°, acquisition time = 1.311 s, no. scans = 2839).
(14) In this experiment the production broth was harvested 193 h after inoculation.
(15) [1-^{13}C]-D-Glucose (99 atom % C-13) was obtained from Omicron Chemicals, Ithaca, NY.
(16) Haslam, E. *The Shikimate Pathway*; Halsted Press: New York, 1974; pp 8-9.
(17) Reference 16, pp 105-107.

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acid precursor relative to **2**, a mixture of [1-^{14}C]-D-erythrose (5.5 μCi), **13a**, and [1-^{13}C]-D-erythrose (336 mg), **13b**,[18] was administered and afforded 8 mg of **2c**. From the ^{13}C NMR spectrum it was clear that **2c** was only labeled at C-1 (5.13%), rather than at C-5. Thus, the nitrogen substituent of **2** had been introduced at C-6 of the shikimate intermediate, rather than at C-2. This is consistent with the involvement of either **4**, **10**,[19] or anthranilic acid, **14**.[20]

S. LL-C10037 was next fermented (2 × 200 mL broths) in the presence of ^{18}O molecular oxygen,[21] yielding 10 mg of **2d**. The ^{13}C NMR spectrum exhibited ^{18}O-induced isotope shifts[22] for C-4 (2.0 Hz), C-5 (3.5 Hz), and C-6 (3.0 Hz), revealing that the secondary alcohol and epoxide oxygens had been introduced by oxidative processes rather than having been retained from the organic precursor. The origin of the C-4 hydroxyl from molecular oxygen ruled out **4** as an intermediate.

[5-^{2}H]-3-Hydroxyanthranilic acid **15**[23] was then fed (200 mg to 200 mL of broth) yielding[24] 5.0 mg of **2e** that was analyzed by ^{2}H NMR.[25] A 67% enrichment of the signal at δ 3.8 (H-5) was observed, representing a 2.2% incorporation of **15** (50 mg was recovered).

The biosynthesis of **2** represents a novel aromatic amino acid metabolism. In view of our work, metabolites such as paulomycin A,[26] the enaminomycins,[27] MM14201,[28] and G7063-2[29] are most likely also derived from the shikimate pathway. We are currently investigating the biosynthesis of the latter two.

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(18) [1-^{13}C]-D-Erythrose (99 atom % C-13) was obtained from Omicron Chemicals, Ithaca, NY.
(19) Reference 16, pp 26–29.
(20) Reference 16, pp 138–139, 160–164.
(21) 50% $^{18}O_2$ was obtained from Cambridge Isotopes, Inc., Woburn, MA.
(22) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252. Vederas, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 374. Vederas, J. C. *J. Chem. Soc., Chem. Commun.* **1980**, 183.
(23) Obtained by exchange (86%) with deuteriotrifluoroacetic acid at 80 °C in a sealed tube for 48 h. In the ^{1}H NMR, the dd at δ 7.44 was nearly gone and the signals at δ 7.33 and 7.82, previously doublets, were now singlets.
(24) The fermentation was harvested 135 h after innoculation.
(25) Spectrum obtained at 61.4 MHz (sweep width = 952 Hz, 4 K data points zero filled to 8 K, pulse width = 90°, acquisition time = 21.5 s, no. scans = 3000).
(26) Wiley, P. F.; Mizsak, S. A.; Baczynskyj, L.; Argoudelis, A. D. *J. Antibiot.* **1984**, *37*, 1273.
(27) Itoh, Y.; Haneishi, T.; Arai, M.; Hata, T.; Aiba, K.; Tamura, C. *J. Antibiot.* **1978**, *31*, 838.
(28) Box, S. J.; Gilpin, M. L.; Gwynn, M.; Hanscomb, G.; Spear, S. R.; Brown, A. G. *J. Antibiot.* **1983**, *36*, 1631. On the basis of optical rotations, this appears to be the enantiomer of **2**.
(29) Noble, M.; Noble, D.; Sykes, R. B. *J. Antibiot.* **1977**, *30*, 455.

Mechanism-Based Isocoumarin Inhibitors for Trypsin-like Serine Proteases Involved in Blood Coagulation†

Chih-Min Kam, J. Clay Copher, and James C. Powers*

School of Chemistry, Georgia Institute of Technology Atlanta, Georgia 30332

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Human plasma contains a number of proteins (zymogens) which are precursors of serine proteases with trypsin-like specificity. The interaction of activated serine proteases with these zymogens and with natural plasma protease inhibitors in a cascade of enzymatic reactions forms the basis of the blood coagulation pathway. Although intravascular clotting is a major health problem in the United States, almost no new anticoagulant drugs have been developed in recent years. A number of heterocyclic compounds have previously been shown to be suicide substrates of serine proteases.[1-6] Substituted isocoumarins have been reported to be mechanism-based inhibitors for elastases and a variety of chymotrypsin-like enzymes.[4,5] Here we report the synthesis of three new isocourmarins with basic functional groups (guanidino or isothiureidoethoxy) attached to the isocoumarin ring system. These compounds are potent inhibitors of coagulation enzymes and are the first effective mechanism-based inhibitors of clotting in human plasma.

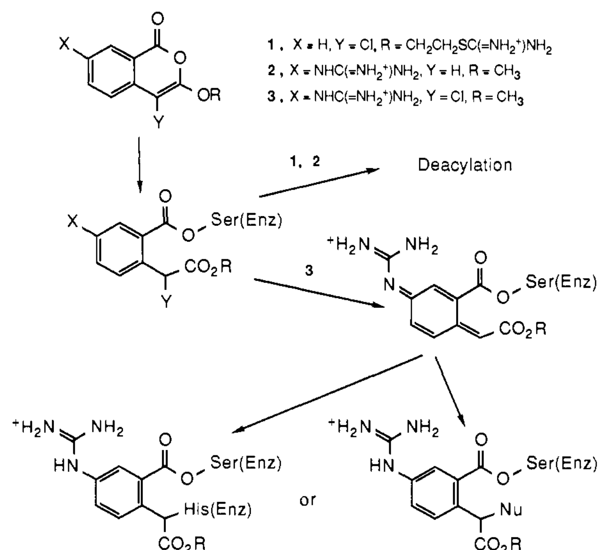

Figure 1. Proposed mechanism of inactivation of serine proteases by substituted isocoumarins.

Incubation of 4-chloro-3-(2-isothiureidoethoxy)isocoumarin (**1**), 7-guanidino-3-methoxyisocoumarin (**2**), and 4-chloro-7-guanidino-3-methoxyisocoumarin (**3**)[7] with several coagulation enzymes and trypsin results in time-dependent loss of enzymatic activity (Table I). Human protein D, human leukocyte elastase, human leukocyte cathepsin G, porcine pancreatic elastase, chymotrypsin, and human plasma plasmin were also inactivated by compound **3** with $k_{obsd}/[I]$ values of 120, 6400, 11000, 860, 7200, and 3600 $M^{-1}s^{-1}$, respectively. Compound **3** was quite selective among serine proteases and has its most potent inhibitory activity toward trypsin, thrombin, and human plasma kallikrein with $k_{obsd}/[I]$ values of $2-3 \times 10^5$ M^{-1} s^{-1}. It was an order of magnitude

† Dedicated to Professor George Büchi on the occasion of his 65th birthday.

(1) Westkaemper, R. B.; Abeles, R. H. *Biochemistry* **1983**, *22*, 3256–3264.
(2) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1983**, *258*, 15046–15053.
(3) Hedstrom, L.; Moorman, A. R.; Dobbs, J.; Abeles, R. H. *Biochemistry* **1984**, *23*, 1753–1759.
(4) Harper, J. W.; Hemmi, K.; Powers, J. C. *Biochemistry* **1985**, *24*, 1831–1841.
(5) Harper, J. W.; Powers, J. C. *Biochemistry* **1985**, *24*, 7200–7213.
(6) Gelb, M. H.; Abeles, R. H. *J. Med. Chem.* **1986**, *29*, 585–589.
(7) The precursor of the isocoumarin, 2-bromoethyl 2-carboxyphenylacetate was synthesized from homophthalic acid and 2-bromoethanol. Methyl 2-carboxy-4-guanidinophenylacetate was prepared from guanidination of methyl 4-amino-2-carboxyphenylacetate with 3,5-dimethylpyrazole-1-carboxamidine nitrate by a standard method described previously: Tsunematsu, H.; Makismi, S. *J. Biochem.* **1980**, *88*, 1773–1783. 3-(2-Bromoethoxy)-4-chloroisocoumarin and compound **2** were prepared by cyclization of the corresponding ester with PCl_5 according to the previous method: Tirodkar, R. B.; Usganokar, R. N. *Indian J. Chem.* **1969**, *7*, 1114–1116. Compound **1** was obtained by refluxing 1 equiv of 3-(2-bromoethoxy)-4-chloroisocoumarin with 2.5 equiv of thiourea in THF for 2 days. Compound **3** was obtained by chlorination of 1 equiv of compound **2** with 1.1 equiv of *N*-chlorosuccinimide in DMF at room temperature for 24 h. Satisfactory NMR, UV, mass spectra, and elementary analysis were obtained for all new compounds. Additional experimental details are given in the Supplementary Material.

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