SYNTHESIS OF N-[3-OXO-(4,5-$^3H_2$)-HEXANOYL] HOMOSERINE LACTONE: BIOLOGICALLY ACTIVE TRITIUM-LABELLED VIBRIO FISCHERI AUTOINDUCER

H. B. Kaplan[1], A. Eberhard[2], C. Widrig[2+], and E. P. Greenberg[1*]

Department of Microbiology, College of Agriculture and Life Sciences

Cornell University, Ithaca, NY 14853[1] and

Department of Chemistry, Ithaca College, Ithaca, NY 14850[2]

SUMMARY

N-(3-oxo-4-hexenoyl) homoserine lactone was synthesized and then reduced with Wilkinson's catalyst in the presence of tritium gas to form N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone (45-55 Ci/mmol). The labelled compound was indistinguishable from the autoinducer for Vibrio fischeri luciferase [N-(3-oxohexanoyl) homoserine lactone] by high-performance liquid chromatography and it was biologically active.

Key Words: Autoinducer, N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone, Bioluminescence, Vibrio fischeri, Wilkinson's catalyst

INTRODUCTION

Luciferase, which catalyzes light emission, is inducible in many luminous bacteria. The inducer (termed autoinducer) is produced by the bacteria and excreted into the culture medium. When the autoinducer reaches a critical extracellular concentration, induction of bacterial luciferase synthesis and thus luminescence commences (1,2). The only autoinducer structure known is that of Vibrio fischeri. This autoinducer, N-(3-oxohexanoyl) homoserine lactone (3), is species-specific: other luminous bacterial species exhibit similar regulatory mechanisms but their autoinducers do not cross-react

---

*Corresponding author

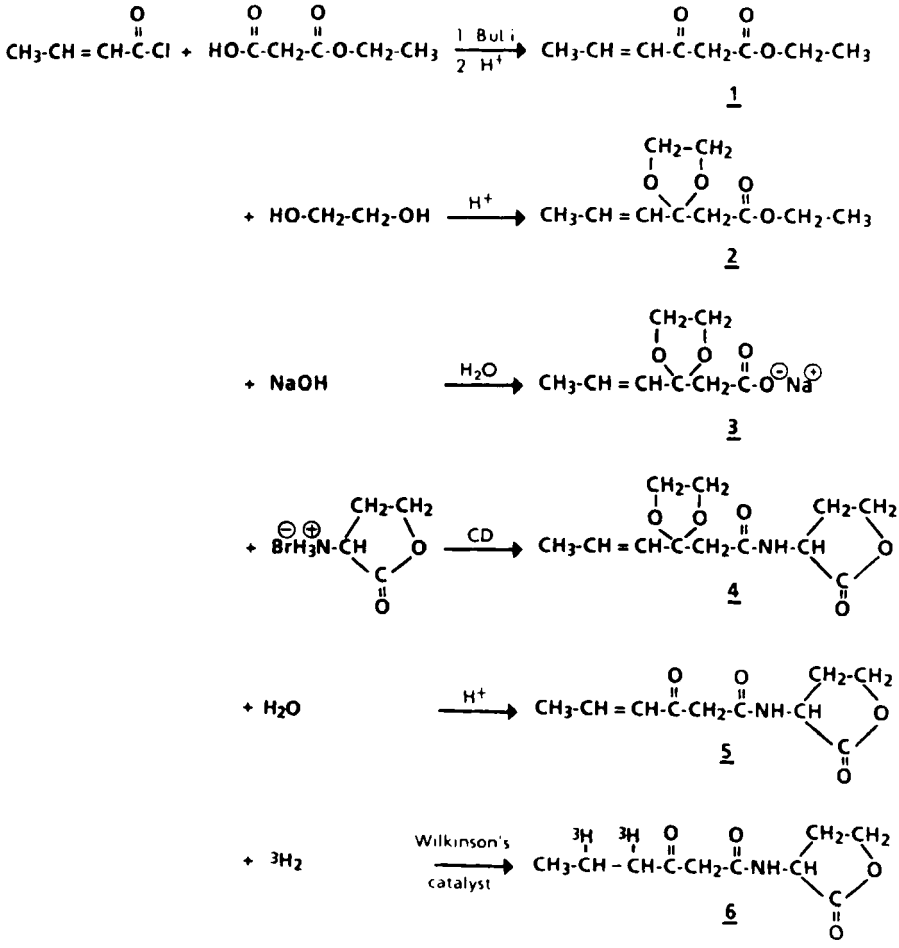
+Present address: Department of Chemistry, University of California, Berkeley, CA 94720

0362-4803/85/040387-09$01.00

Received August 8, 1984
Revised September 28, 1984

with that produced by V. fischeri (1,4). Autoinduction can be considered an environmental sensing mechanism: in habitats where a species-specific autoinducer can accumulate the bacteria are signalled to synthesize luciferase (2). Autoinduction in V. fischeri is thought to be of ecological importance since it ensures luciferase synthesis when cells are in a symbiotic habitat, such as the light organs of pinecone fish, where they are contained at high densities (5,6,7).

To learn more about the phenomenon of autoinduction and to understand better the control of genes expressed specifically when bacteria are in symbi-



Scheme for the synthesis of N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone.

otic associations with higher organisms, we synthesized radiolabelled autoinducer of high specific activity for use in tracer studies. One advantage of our method of synthesis is that radioisotopes were not involved until the final reaction step. An unsaturated analogue of autoinducer [N-(3-oxo-4-hexenoyl) homoserine lactone 5] was synthesized, and then reduced with carrier-free tritium gas by Wilkinson's catalyst to form the labelled autoinducer, 6.

EXPERIMENTAL

Reagents. Tris (triphenylphosphine) rhodium (I) chloride (Wilkinson's homogeneous catalyst [8]), α-amino-γ-butyrolactone hydrobromide, crotonic acid, diethyl malonate and n-butyl lithium were obtained from Aldrich Chemical Co., Milwaukee, WI. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from Sigma Chemical Co., St. Louis, MO. Dowex 50W-X4 and Dowex 1-X4 50-100 mesh ion exchange resins were obtained from Bio-Rad Laboratories, Richmond, CA.

Apparatus. Generally, the equipment for high-performance liquid chromatography (HPLC) included a Beckman 110A pump, an Altex 156 refractive index detector and a 1 cm x 25 cm C_{18} reverse phase column (Altex Ultra-sphere ODS). Preparative HPLC of radiolabelled autoinducer 6 was performed at New England Nuclear Corp., Boston, MA, with a Waters M600A pump, a Waters 660 solvent programmer, a 2 cm x 50 cm C_{18} reverse phase column (Whatman M20, Partisil-10 ODS-3), and a modified flow through Geiger counter as the detector. A Varian T-60 NMR spectrometer was used to obtain ^1H NMR spectra. Internal or external standards of tetramethyl silane were used to determine chemical shifts. Infrared spectra were obtained with a Perkin Elmer 137 IR spectrophotometer. Mass spectra were obtained with an AEI MS902 mass spectrometer. A Perkin-Elmer 151 annular still was used for spinning band distillations. Bacterial luminescence was monitored with a Turner model 20 photometer adjusted for continuous light measurement, and calibrated as previously described (9). Standard procedures were used for scintillation counting.

Bacterial strain and growth medium. The bacterial strain used was V. fischeri B61 (2,10). This strain makes very little autoinducer and therefore is

dim. Exogenously added autoinducer stimulates luminescence of strain B61, making this an appropriate strain for use in autoinducer bioassays (2). The culture medium contained the following ingredients per liter: 5 g Tryptone (Difco, Detroit, MI), 3 g Yeast Extract (Difco, Detroit, MI), 3 ml glycerol, 50 mmol Tris base, 19 mmol NH_4Cl, 0.33 mmol $K_2HPO_4 \cdot 3H_2O$, 1.8 nmol $FeSO_4 \cdot 7H_2O$, and 500 ml of seawater salts solution (seawater salts solution consisted of 0.6 M NaCl, 0.1 M $MgSO_4 \cdot 7H_2O$, 0.02 M $CaCl_2 \cdot 2H_2O$, and 0.02 M KCl). The medium was brought to volume with deionized water. The pH was adjusted to 7.0 with concentrated HCl and the medium was sterilized by autoclaving.

Autoinducer bioassays. Cells used for bioassays were cultured in 5 ml of medium contained in 16 x 150 mm tubes. Incubation was at 22°C with shaking. The cultures were inoculated with 0.2 ml of a mid-logarithmic phase culture which had been stored in liquid nitrogen. Cells were harvested by centrifugation (8,000 x g at 3°C for 5 min) when cultures reached a density of 1.0 (OD_{660} Spectronic 21, Bausch and Lomb, Rochester, NY). Cells from a 5-ml culture were resuspended in 50 ml of bioassay medium (0.3 M NaCl, 0.05 M $MgSO_4 \cdot 7H_2O$, 0.01 M potassium phosphate buffer, [pH 7.0], 0.05% Tryptone [Difco, Detroit, MI], and 0.03% glycerol). This cell suspension (approximately 3 x 10^8 cells per ml) was stored on ice for use in bioassays.

For each autoinducer bioassay, 1 ml of the cell suspension described above was dispensed into a 20-ml glass scintillation vial and the compound tested was added as a solution in 50 µl or less of deionized water. Incubation was at 25°C and luminescence was measured periodically.

Ethyl 3-oxo-4-hexenoate (1)

Crotonyl chloride was obtained as a distillate after refluxing crotonic acid with thionyl chloride. Monoethyl hydrogen malonate was prepared by KOH hydrolysis of diethyl malonate in ethanol and subsequent acidification with HCl (11). This was converted to the dilithio dianion with 2 equivalents of n-butyl lithium in tetrahydrofuran. Crotonyl chloride

and the dilithio dianion of monoethyl hydrogen malonate were then allowed to react to give a 24% yield of the crude β-keto ester, ethyl 3-oxo-4-hexenoate 1 (12).

Ethylene glycol ketal of ethyl 3-oxo-4-hexenoate (2)

To prevent decarboxylation in later steps, 1 was protected through formation of its ethylene glycol ketal by refluxing 10.2 g of 1 with 12.2 g of ethylene glycol and 0.115 g of p-toluene sulfonic acid·H_2O in 250 ml of benzene for 28 h. A Dean-Stark trap was used to monitor the progress of this reaction. Upon completion of the reaction, the mixture was washed with 50 ml of a saturated $NaHCO_3$ solution and 50 ml of a saturated NaCl solution, dried over $MgSO_4$ and the solvent removed by rotary evaporation. This was performed three times and the products were pooled and purified by spinning band distillation, giving a 35% yield of 2 boiling at 68-69°C/0.2 torr; ^{1}H NMR (CCl_4): 1.2(t,3H,-O-CH_2-CH_3), 1.8(d,3H,CH_3-CH=), 2.6(s,2H,-CH_2-COO-), 4.0(s,4H, ketal ring hydrogens), 4.2(q,2H,-O-CH_2-CH_3), 5.7-6.2(m,2H,CH_3-CH=CH-).

Ethylene glycol ketal of sodium 3-oxo-4-hexenoate (3)

The protected β-keto ester 2 was converted to its sodium salt 3 by heating at reflux for 1 h with a slight excess of NaOH. The reaction product was neutralized with HCl, washed three times with diethyl ether, and then water was removed by rotary evaporation.

Ethylene glycol ketal of N-(3-oxo-4-hexenoyl) homoserine lactone (4)

A mixture of 48.25 mmol of 3 was stirred with a slight excess of α-amino-γ-butyrolactone hydrobromide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 75 ml of water for 24 h. The water was removed by rotary evaporation and absolute ethanol was added to the thick yellow oil which remained. The resulting yellow solution was separated from the white precipitate and the ethanol was removed by rotary evaporation leaving a yellow oil. This oil was repeatedly extracted by trituration in warm ethyl acetate. The solutions were pooled and the ethyl acetate was removed by rotary evaporation. The remaining material was dissolved in water

and purified by Dowex 50W-X4 cation exchange and Dowex 1-X4 anion exchange column chromatography giving 30.2 mmol (63%) 4; ^{1}H NMR (D_2O): 2.0(d,3H,CH_3-CH=), 3.1(s,2H,-CH_2-COO-), 4.3(s,4H, ketal ring hydrogens), 5.7-6.7(m,2H,CH_3-CH=CH-), 2.6-3.0, 4.6-5.2 (m,5H, lactone ring hydrogens); IR: (1770, 1720, 1660, 1540, 1180, 1030 cm^{-1} major bands).

N-(3-oxo-4-hexenoyl) homoserine lactone (5)

Compound 4 (30.2 mmol) was deprotected by refluxing in 100 equivalents of water at pH 4.0 for 5 min. The water was removed by rotary evaporation and the remaining product was purified by HPLC using water: methanol, 65:35 at a flow rate of 2.0 ml per min. Each sample injected contained approximately 100 mg of 5. Yield: 76%. Mass spectra exhibited the molecular ion at m/e 211.0828. This corresponded to the elemental composition $C_{10}H_{13}NO_4$ (calculated value, 211.0844).^{1}H NMR(D_2O): 2.1(d,3H,CH_3-CH=), 3.9(s,2H,-CH_2-CON-), 6.3-6.6(d,1H,CH_3-CH=CH-), 7.1-7.7(m,1H,CH_3-CH=), 2.4-3.2, 4.5-5.1(m, 5H, lactone ring hydrogens).

N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone (6)

The selective reduction of 5 to give the tritiated autoinducer 6 was accomplished by dissolving 20 mg (0.095 mmol) of 5 in 1 ml of benzene: ethanol (1:1), adding 5 mg of Wilkinson's homogeneous catalyst (8,13) and stirring overnight at room temperature in an atmosphere of tritium gas (85 Ci). During the reaction, 2.3 ml of tritium gas was absorbed. Labile tritium was removed *in vacuo* with a solution of benzene: ethanol (1:1, 6 ml). The residue was dissolved in 2 ml of methanol, sonicated for 15 min and filtered. The filter was washed with 3 ml of methanol and the combined methanol solutions were evaporated to dryness *in vacuo*. The resulting oil was triturated twice in 2 ml of water. The water was removed by rotary evaporation and the crude 6 was purified by preparative HPLC using water: methanol, 75:25 at a flow rate of 7.5 ml per min. There was a sharp peak of radioactivity in the 427.5 to 450 ml fraction of eluant. This peak was collected, the solvent removed by evaporation and the remaining

radioactive material dissolved in ethyl acetate, giving 547 mCi of tritium. This corresponds to approximately 0.011 mmol (12% yield) of N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone, 6 (45-55 Ci/mmol). The selective reduction step and purification of 6 were performed by the Tritium/Radioiodine Labeling Services Department at New England Nuclear Corp., Boston, MA.

Evidence to support the structural assignment for the synthesized compound 6 was obtained by HPLC analysis (Fig. 1). Furthermore, the biological activity of the radiolabelled compound 6 was similar to that of autoinducer,

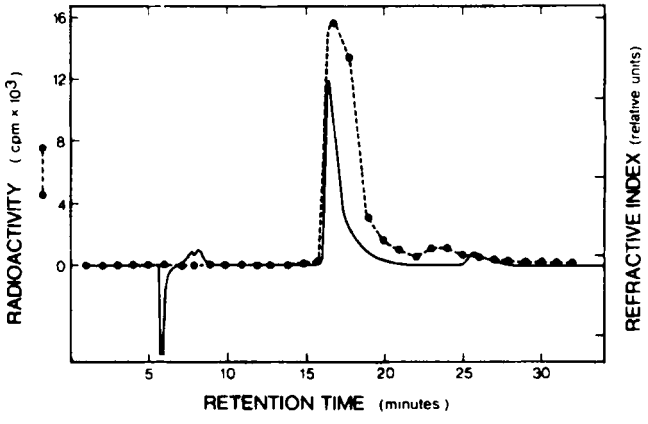


Figure 1. Chromatographic comparison of the radiolabelled compound 6 with autoinducer, N-(3-oxohexanoyl) homoserine lactone. A sample containing 19 nmol of unlabelled autoinducer and 0.1 μCi (2 pmol) of the radiolabelled compound was subjected to HPLC using water: methanol, 75:25 at a flow rate of 2 ml per min. The eluant was collected in 2 ml fractions and radioactivity was determined using liquid scintillation counting procedures.

The slight radiochemical impurity that began to appear at ca 23 minutes constitutes <10% of the total radioactivity. Rechromatography of autoinducer separated from the impurity by HPLC always showed a small peak with a retention time in the range of 23-26 minutes (data not shown). Thus we assume this impurity is an equilibrium product or products formed from autoinducer.

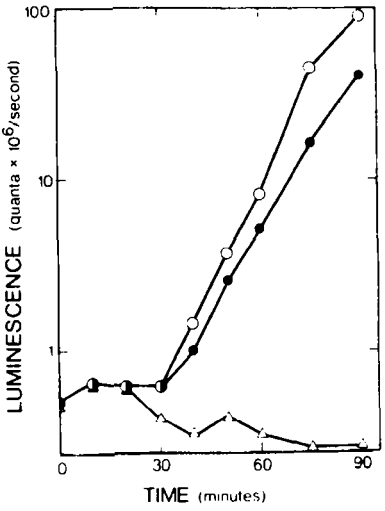


Figure 2. Bioassay for autoinducer activity of N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone. Positive control, 0.2 nmol of unlabelled autoinducer added to V. fischeri cells, (o); negative control, no additions, (Δ); 10 μCi (0.2 nmol) of N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone added, (●).

N-(3-oxohexanoyl) homoserine lactone (Fig. 2). Specific activity of compound 6 was calculated from the results of several bioassays (data not shown) to fall within a range of 45-55 Ci/mmol. The mean value was 50 Ci/mmol.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Cornell Biotechnology Program which is sponsored by the New York State Science and Technology Foundation and a consortium of industries, the Research Corporation (A.E. and C.W.) and USDA/Hatch. The mass spectral analysis was performed by the Mass Spectrometry Facility at Cornell University. The authors thank Katharine Bradley of New England Nuclear Corp.

REFERENCES

1. Eberhard A. - J. Bacteriol. 109:1101 (1972)
2. Nealson K.H. - Arch. Microbiol. 112:73 (1977)
3. Eberhard A., Burlingame A.L., Eberhard C., Kenyon G.L., Nealson K.H. and Oppenheimer N.J. - Biochemistry 20:2444 (1981)
4. Greenberg E.P., Hastings J.W. and Ulitzur S. - Arch. Microbiol. 120:87 (1979)
5. Ruby E.G. and Nealson K.H. - Biol. Bull. 151:574 (1976)
6. Fitzgerald J.M. - Arch. Microbiol. 112:153 (1977)
7. Ruby E.G. and Morin J.G. - Deep-Sea Res. 25:161 (1978)
8. Young J.F., Osborn J.A., Jardine F.H. and Wilkinson G. - Chem. Comm. 131 (1965)
9. Hastings J.W. and Weber G. - J. Opt. Soc. Amer. 53:1410 (1963)
10. Baumann P., Baumann L. and Mandel M. - J. Bacteriol. 107:268 (1971)
11. Strube R.E. - Org. Synth. Coll. 4:417 (1963)
12. Wierenga W. and Skulnick H.I. - J. Org. Chem. 44:310 (1979)
13. Harmon R.E., Parsons J.L., Cooke D.W., Gupta S.K. and Schoolenberg J. - J. Org. Chem. 34:3684 (1969)