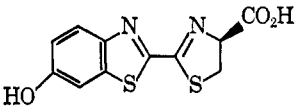
Amino Analogs of Firefly Luciferin and Biological Activity Thereof[1]

Emil H. White, Helmut Wörther, Howard H. Seliger, and William D. McElroy

Contribution from the Department of Chemistry and the McCollum-Pratt Institute and Department of Biology of the Johns Hopkins University, Baltimore, Maryland 21218. *Received January 7, 1966*

Abstract: For our study of the mechanism of firefly bioluminescence, the compounds 2-(6′-amino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (IVa, "aminoluciferin"), 2-(6′-acetylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (IVb), and 2-(6′-trifluoroacetylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (IVc) have been synthesized and characterized. Of these compounds, only aminoluciferin reacts with firefly luciferase and ATP to produce light. The bioluminescence emission is red and is pH independent, unlike the reaction with natural luciferin, which emits in the yellow-green at neutral and alkaline pH and in the red at acid pH. The fluorescence of aminoluciferin is compared with that of native luciferin.

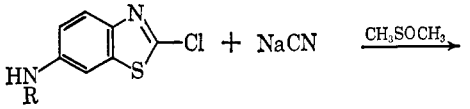
Native firefly (*Photinus pyralis*) luciferin has been shown to be D-(−)-2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (I) by degradation and total synthesis.[2] Only the D-(−) isomer of firefly luciferin is biologically active in the production of light; the L-(+)-luciferin will react with firefly luciferase



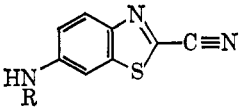
I

and adenosine triphosphate (ATP) in the presence of Mg^{2+} ions to form the intermediate E·LH_2AMP and to release inorganic pyrophosphate, but the subsequent reaction with molecular oxygen does not occur.[3,4] It is further known that the color of the *in vitro* bioluminescence reaction of luciferin and luciferase is markedly dependent upon the pH of the reaction solution.[5] As shown in Figure 1 there appear to be two different emitting species, one leading to the normal yellow-green emission at neutral and alkaline pH, peaking at 562 mμ, and identical with that observed in the live firefly, and the second yielding a red emission at acid pH, peaking at 614 mμ. These curves are normalized at their respective peak wavelengths; otherwise the red emission would be very much lower in intensity since the red bioluminescence quantum yield is very much lower than the yellow-green bioluminescence quantum yield. In view of the fact that the decrease in yellow-green emission and the relative increase in red emission were also observed for an increase in temperature of the reaction above 23° and in the presence of urea, Zn^{2+}, Cd^{2+}, or Hg^{2+} ions, of which temperature and urea have been demonstrated in other enzyme systems to effect changes in enzyme configuration as evidenced by optical rotatory dispersion, it was hypothesized that the configuration of the enzyme, aside from influencing the rate of the reaction, allowed other groups on the enzyme to interact with the chemically excited product molecule to produce essentially a different emitting species. The question therefore arose as to the function of the 6′-OH group of the luciferin molecule, both in biological activity and in the nature of the bioluminescence emission, but separate from the D–L stereospecificity.

For this reason, three amino analogs of D,L firefly luciferin (IVa–c) were synthesized; both the details of synthesis and the biological activity are described in this paper. The method of preparation was a modification of that previously used for the synthesis of firefly luciferin. Nitration of 2-chlorobenzothiazole and reduction of the nitro derivative by the method of Katz[6] yielded 6-amino-2-chlorobenzothiazole (IIa); acylation then readily yielded derivatives IIb and IIc. The reaction of these compounds with potassium cyanide in dimethyl sulfoxide yielded the corresponding nitriles (IIIa–c). Lastly, condensation of the nitriles with



IIa, R = H
b, R = CH_3CO
c, R = CF_3CO



IIIa
b
c

cysteine yielded 2-(6′-amino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (aminoluciferin, IVa), 2-(6′-acetylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (acetylaminoluciferin, IVb), and 2-(6′-trifluoroacetylamino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (trifluoroacetylaminoluciferin, IVc), respectively. The luciferins are difficult compounds to purify, and as a result the trifluoroacetyl derivative

(1) For the synthesis of other analogs of firefly luciferins, see: (a) E. H. White, H. Wörther, G. F. Field, and W. D. McElroy, *J. Org. Chem.*, **30**, 2344 (1965); (b) E. H. White and H. Wörther, *ibid.*, **31**, 1484 (1966). Contribution No. 466 of McCollum-Pratt Institute.

(2) E. H. White, F. McCapra, G. F. Field, and W. D. McElroy, *J. Am. Chem. Soc.*, **83**, 2402 (1961); E. H. White, F. McCapra, and G. F. Field, *ibid.*, **85**, 337 (1963).

(3) H. H. Seliger, W. D. McElroy, E. H. White, and G. F. Field, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1129 (1961).

(4) W. D. McElroy and H. H. Seliger, *Advan. Enzymol.*, **25**, 119 (1963).

(5) H. H. Seliger and W. D. McElroy, *Arch. Biochem. Biophys.*, **88**, 136 (1960).

(6) L. Katz, *J. Am. Chem. Soc.*, **73**, 4007 (1951).

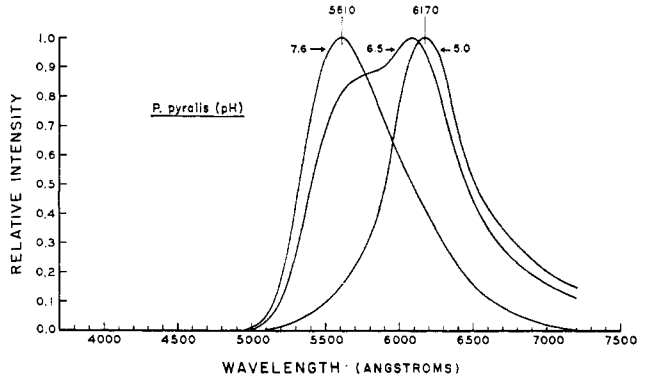


Figure 1. Effect of pH on the *in vitro* bioluminescence emission of *P. pyralis* luciferase plus *P. pyralis* luciferin (I). Reaction initiated with ATP and Mg^{2+} ions in 0.025 *M* glycylglycine buffer. The spectra are normalized to 1 at the peak emission.

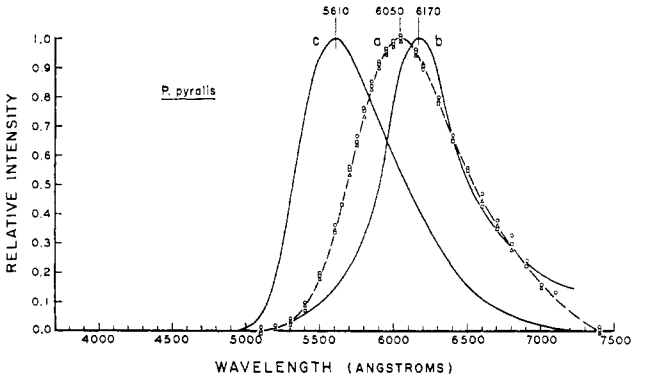
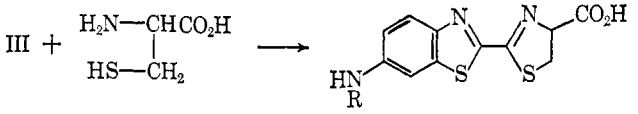


Figure 2. Bioluminescence emission spectra of (a) *P. pyralis* luciferase plus 6′-NH_2LH_2 at pH 6.0 (○), pH 7.7 (□), and pH 8.55 (△); (b) *P. pyralis* luciferase plus luciferin (I) at pH 6.0 and (c) *P. pyralis* luciferase plus luciferin (I) at pH 7.6. The spectra are normalized to 1 at peak emission.

(IVc) was not obtained analytically pure; the ultraviolet and infrared spectra indicated that our material was largely IVc, however.



IVa, R = H
b, R = CH_3CO
c, R = CF_3CO

The ultraviolet spectra of these compounds are listed in Table I. The amino derivatives absorb at considerable longer wavelengths than the corresponding hydroxy compounds. A similar trend is noted in the spectrum of *p*-nitroaniline (375 mμ) as compared to that of *p*-nitrophenol (314 mμ) in ethanol.[7]

Table I. Ultraviolet Spectra of the Aminobenzothiazoles (III) and the Aminoluciferins (IV)

Compound	Absorption band, λ_{max} mμ (log ε)[a]		
IIIa	241 (3.83)	277 (3.80)	365 (4.19)
IIIb	218 (4.10)	254 (3.70)	323 (3.95)
2-Cyano-6-hydroxy-benzothiazole		263 (3.90)	322 (4.21)
IVa	220 (4.41)	280 (3.78)	363 (4.19)
IVb	225 (4.44)		327 (4.33)
I		269 (3.85)	330 (4.26)

[a] Measured in 95% ethanol.

The infrared spectrum in KBr of aminoluciferin (IVa), which shows from the weakness of the 5.80-μ band and the strength of the 6.30-μ band that the molecule exists largely in the zwitterionic form, is quite different from that of firefly luciferin (I) itself. Common bands, however, are medium to strong ones at 9.5–9.6, 10.8–10.9, 11.3–11.4, and 12.6–12.8 μ. The infrared spectrum of the acetyl derivative (IVb) also contains strong bands at the first three positions.

Hydrolysis of IVb led to a large number of degradation products; thus aminoluciferin (IVa) was prepared directly *via* the chloroaminobenzothiazoles IIa and IIIa. Trifluoroacetylaminoluciferin (IVc) was synthesized to study the effect of a strongly electron-attracting center at the amino group.

Only 2-(6′-amino-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (IVa), of the amino analogs, was found to be effective in producing light when mixed with firefly (*Photinus pyralis*) luciferase, ATP, and Mg^{2+} ions in 0.025 *M* glycylglycine or 0.05 *M* Tris buffer. The emission at neutral and alkaline pH is red, peaking at 605 mμ, very close to the bioluminescence emission of firefly luciferin (I) at acid pH. More significantly the color of the 6′-aminoluciferin bioluminescence is entirely independent of pH, from below 6 to above 10, in exactly the range where native firefly luciferin (I) shows the remarkable color shifts outlined above. Since phenols are stronger acids than anilines, this observation suggests that it is the phenolate ion of firefly luciferin that is involved in the normal yellow-green bioluminescence of I. The data are summarized in Figure 2, which shows the true normalized emission spectra in numbers of photons per second per wavelength interval as a function of wavelength for (a) the superimposed bioluminescence emission spectra of 6′-NH_2LH_2 (IVa) at pH 6.0, 7.7, and 8.55, (b) the bioluminescence emission spectrum of firefly luciferin (I) at pH 6.0, and (c) the bioluminescence emission spectrum of firefly luciferin (I) at pH 7.6. All spectra were measured at 25°.

Biological Assays. The enzyme luciferase was extracted from dried *Photinus pyralis* tails as described previously.[8] The light-emitting reactions were run in 10 × 75 mm glass tubes containing enzyme, Mg^{2+} ions as sulfate, and the luciferin substrate in 0.025 glycylglycine buffer at pH 7.6. The reactions were initiated by injection of excess adenosine triphosphate (ATP) solution (10 mg/ml) with a hypodermic syringe. The subsequent light was observed either visually or the reaction tube was placed at the entrance slit of an f/3 grating photoelectric spectrometer and the spectrum was recorded. The spectral response of the spectrometer phototube combination was measured with a National Bureau of Standards color temperature standard lamp.[9] All reported spectral data are true photon distributions.

(7) "Organic Electronic Spectral Data," Vol. IV, J. P. Phillips, and F. C. Nachod, Ed., Interscience Publishers, Inc., New York, N. Y., 1963.

(8) W. D. McElroy, *Federation Proc.*, **19**, 941 (1960).

(9) J. Lee and H. H. Seliger, *Photochem. Photobiol.*, **4**, 1015 (1965).

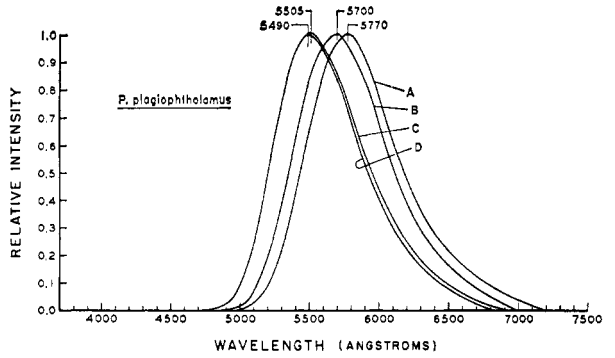


Figure 3. *In vitro* bioluminescence emission spectra of (A) *P. plagiophthalamus* ventral organ luciferase plus luciferin (I), (B) *P. platiophthalamus* ventral organ luciferase plus aminoluciferin (IVa), (C) *P. platiophthalamus* dorsal organ luciferase plus luciferin (I), (D) *P. plagiophthalamus* dorsal organ luciferase plus aminoluciferin (IVa). All reactions run in 0.025 *M* glycylglycine buffer at pH 7.6. The spectra are normalized to 1 at peak emission.

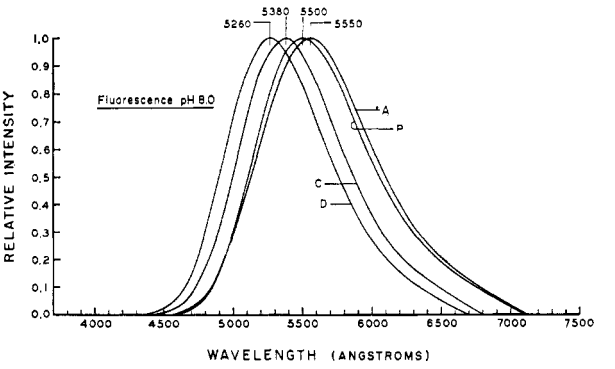
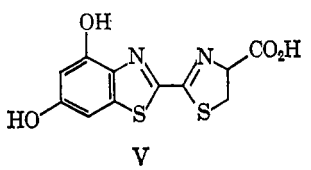


Figure 4. Fluorescence emission spectra of (A) dehydroluciferin (VI), (B) 4′-hydroxyluciferin (V), (C) luciferin (I), (D) aminoluciferin (IVa) in glycylglycine buffer at pH 8.0. Spectra are normalized to 1 at peak emission.

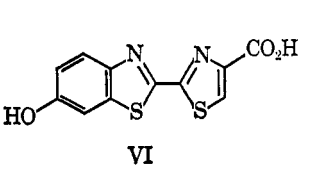
It has already been demonstrated that among the various firefly species whose *in vivo* bioluminescence emission spectra are different from *Photinus pyralis*, namely *Photuris pennsylvanica* and *Pyrophorus plagiophthalamus*, the species enzyme is responsible for the color of the light emission.[10] That is, when the same synthetically prepared D-firefly luciferin (I) is treated with luciferase extracted from the light organs of these fireflies, the *in vitro* bioluminescence emission spectrum matches the original *in vivo* emission spectrum. *P. plagiophthalamus* is unique among the fireflies in possessing two different types of light organs: a pair of dorsal prothoracic light organs emit a bright green bioluminescence and are used for sex location on the ground while a single ventral light organ lights up a bright yellow only in flight. Luciferase extracted from these two different organs gave on incubation with luciferin (I) *in vitro* bioluminescence emission spectra that corresponded to their *in vivo* emission spectra.

In Figure 3 are shown the true normalized emission spectra of *P. plagiophthalamus* dorsal light organ luciferase and ventral light organ luciferase, each allowed to react with 6′-NH_2LH_2 (IVa) and luciferin (I). With the dorsal enzyme at pH 7.6 the aminoluciferin reaction is identical in emission color (peak 550 mμ) with natural luciferin. With the ventral enzyme at pH 7.6 there appears to be a very slight shift for aminoluciferin emission (570 mμ) compared with natural luciferin (577 mμ). All reactions were run in 0.025 *M* glycylglycine buffer at pH 7.6.

In Figure 4 are shown the true normalized fluorescence emission spectra of (D) aminoluciferin (IVa), (C) luciferin (I), (B) 4′-hydroxyluciferin (V)[1b] (the only other derivative of luciferin thus far found to give bioluminescence), and (A) dehydroluciferin (VI) in glycylglycine buffer at pH 8.0 excited at 25° with the 365–366-mμ lines of a mercury arc lamp. The emission peaks ascend in wavelength, 526, 538, 550, and 555 mμ, respectively.



V



VI

In the firefly light reaction the initial step requires the formation of the active intermediate of luciferin, luciferyl adenylate,[11] which then reacts enzymatically with molecular oxygen to produce an electronically excited product molecule. As can be seen from Figures 1 and 4, the normal bioluminescence emission differs only slightly from the fluorescence emission of luciferin (I) or dehydroluciferin (VI). However, both the red bioluminescence emission of luciferin (I) observed at acid pH and the red bioluminescence emission of aminoluciferin (IVa) are markedly different from their respective fluorescences. The large red shift is presumptive evidence of a much greater degree of electron conjugation in the excited species responsible for the light emission. In view of the pH independence of the aminoluciferin bioluminescence reaction, it might be inferred in this instance that the luciferase enzyme configuration has no effect on the degree of conjugation of the excited product molecule, or that the phenol group of luciferin is ionized at a neutral pH, but the amino group of aminoluciferin is in the uncharged form at all pH's tested. However when aminoluciferin (IVa) is treated with *P. plagiophthalamus* dorsal organ luciferase the bioluminescence emission is identical with that from native luciferin (I). Further, a yellow color (5700-A peak; Figure 3B) is observed in the reaction with *P. plagiophthalamus* ventral organ luciferase. This is intermediate between the green (5505-A peak) of the dorsal enzyme reaction and the yellow (5770-A peak) of the ventral enzyme reaction, both with native luciferin. We do not yet understand the nature of the excited species responsible for firefly bioluminescence.

Experimental Section

Aminoluciferin (IVa). A. 2-Chloro-6-aminobenzothiazole (IIa). This compound was prepared from 2-chlorobenzothiazole (Eastman Kodak Co.) by the method of Katz.[6]

B. 2-Cyano-6-aminobenzothiazole (IIIa). Potassium cyanide (450 mg, 6.9 mmoles) was dissolved almost completely in dimethyl sulfoxide (100 ml) by heating the stirred mixture overnight at 130–140° under anhydrous conditions. The temperature of the oil bath was lowered to 120° and 2-chloro-6-aminobenzothiazole (370 mg, 2.00 mmoles) was added in small portions over a period

(10) H. H. Seliger, and W. D. McElroy, *Proc. Natl. Acad. Sci. U. S.* **52**, 75 (1964).

(11) W. C. Rhodes and W. D. McElroy, *Science*, **128**, 523 (1958); *J. Biol. Chem.*, **233**, 1528 (1958).

of 1 hr. The mixture was heated until the ultraviolet peak at 302 mμ of the starting material was replaced by the 364- and 278-mμ absorption maxima of the product; about 4.5 hr was required. After cooling, the mixture was partitioned between a solution of potassium dihydrogen phosphate (0.2 *M*, 250 ml) and ether (500 ml). The ether was separated and the extraction was repeated using 250 ml of ethyl acetate. The organic extract was washed with water (100 ml) and the solvent was removed *in vacuo* after drying over sodium sulfate. The residue was dissolved in ethyl acetate (40 ml), silica gel (5 g) was added, and the solvent was removed *in vacuo*. The dry material was added to the top of a column of silica gel prepared in petroleum ether. The elution was started with 10% ethyl acetate in petroleum ether. A blue fluorescent band was eluted which contained only a small amount of material (discarded). Elution was continued with 200 ml of 20% ethyl acetate and 100 ml of 30% ethyl acetate. The next 460 ml contained the product as shown by the ultraviolet absorption at 363 and 278 mμ. Evaporation yielded 212 mg (1.21 mmoles, 60.5%) of 2-cyano-6-aminobenzothiazole. For analysis, this material was recrystallized from chloroform to yield yellow crystals, mp 216–218 (sinter 212°).

Anal. Calcd for $C_8H_5N_3S$: C, 54.84; H, 2.88; N, 23.99; S, 18.30. Found: C, 54.71, 54.88; H, 2.81, 2.93; N, 24.08, 24.06; S, 18.03, 18.15.

C. 2-(6′-Amino-2′-benzothiazolyl-Δ²-thiazoline-4-carboxylic Acid (IVa). D,L-Cystine (45 mg, 0.187 mmole) was dissolved in liquid ammonia and sodium was added in small pieces until the blue color persisted for 10 min. The flask was flushed with nitrogen and the excess sodium was destroyed with ammonium chloride. The ammonia was removed in a stream of nitrogen. The dry residue was dissolved in water (10 ml, flushed with nitrogen) and the pH was adjusted to about 7.5. Methanol (10 ml, flushed with nitrogen) was added and a solution of 2-cyano-6-aminobenzothiazole (70 mg, 0.400 mmole) in methanol (5 ml) was added to the mixture with shaking. The yellow-green solution of the nitrile changed to a deeper shade. The mixture was allowed to react in the dark for 2 hr. Aliquots were removed after 5 and 100 min and diluted with ethanol; these had absorption maxima at 335 and 278 mμ.

The mixture was diluted with an equal volume of water (flushed with nitrogen), the pH was adjusted to 8.5, and the larger part of the methanol was evaporated *in vacuo*. The resulting solution was extracted with ethyl acetate (100 ml, flushed with nitrogen), and the aqueous layer remaining (30 ml) was acidified. The solution was extracted with ethyl acetate (100 ml), and the extract was dried and the solvent was removed *in vacuo*. The residue consisted of 97 mg (0.348 mmole, 93%) of pure 2-(6′-amino-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylic acid. For analysis, this material was dissolved first in an excess of ethyl acetate and acetone (flushed with nitrogen). The solution was then concentrated under nitrogen and some cyclohexane was added. There resulted a yellowish precipitate of aminoluciferin which melted with decomposition at 210–220°. The crude material proved to be almost as pure as the analytical sample when checked by paper chromatography.

Anal. Calcd for $C_{11}H_9N_3O_2S_2$: C, 47.30; H, 3.25; N, 15.05; S, 22.97. Found: C, 47.41; H, 3.40; N, 14.79; S, 23.10.

N-Acetylaminoluciferin (IVb). A. 2-Chloro-6-acetamidobenzothiazole (IIb). 2-Chloro-6-aminobenzothiazole (IIa, 1.32 g, 7.15 mmoles) was dissolved in 13 ml of acetic anhydride. Pyridine (3 ml) was added dropwise and the solution was slowly raised to the boiling point. After 5 min of heating, the solution was cooled and poured into water. The resulting oily layer was separated; on standing it crystallized to yield 1.33 g (5.87 mmoles, 82%) of the acetamidochlorobenzothiazole, mp 133–134° (lit.[6] mp 130–131°).

B. 2-Cyano-6-acetylaminobenzothiazole (IIIb). Potassium cyanide (95 mg, 1.46 mmoles) was heated with dimethyl sulfoxide (25 ml) for 15 min at 130°. 2-Chloro-6-acetamidobenzothiazole (280 mg, 1.23 mmoles) was added and the reaction mixture was heated at 130° for 2 hr. The mixture was allowed to stand overnight and then it was partitioned between a solution of potassium dihydrogen phosphate (pH *ca.* 4, 150 ml) and ether (250 ml). The extraction of the aqueous layer was repeated using a 100-ml portion of ether. The solvent layer was washed two times with water (50 ml each) and then dried over sodium sulfate. Silica gel was added, the solvent was removed *in vacuo*, and the dry residue was slurried on top of a column of silica gel with petroleum ether. The product was eluted with ether–petroleum ether mixtures containing up to 50% ether. The ultraviolet N absorption of the product (λ_{max} 323 mμ) was used to detect the main fraction. This main fraction contained 209 mg (0.96 mmole, 78%) of 2-cyano-6-acetaminobenzothiazole, mp 237.5–241°. For analysis, this product was recrystallized from ethyl acetate to give yellow needles, mp 241.5–243°.

Anal. Calcd for $C_{10}H_7N_3OS$: C, 55.28; H, 3.25; N, 19.35; S, 14.76. Found: C, 55.21; H, 3.27; N, 19.24; S, 14.86.

C. 2-(6′-Acetylamino-2′-benzothiazolyl-Δ²-thiazoline-4-carboxylic Acid (IVb). D,L-Cystine (121 mg, 0.503 mmole) was reduced as described above with sodium in liquid ammonia under nitrogen. The dry residue after evaporation was dissolved in water (10 ml, flushed with nitrogen) and the pH of the solution was adjusted to about 7.6. Methanol (20 ml) and ethyl acetate (10 ml) were added and to the mixture was added a solution of 2-cyano-6-acetylaminobenzothiazole (239 mg, 1.102 mmoles) in a hot mixture of methanol (10 ml) and ethyl acetate (5 ml). Any residue was dissolved in hot ethyl acetate (10 ml) and also added to the reaction flask. The addition of the nitrile took about 5 min. The mixture was allowed to react for 1 hr. Aliquots were tested by paper chromatography and by ultraviolet absorption. The mixture was diluted with water (50 ml) and 1 ml of 2 *N* sodium hydroxide was added. The alkaline mixture was extracted two times with ethyl acetate (100 ml each) and the aqueous solution was filtered into a dust-free flask. On acidification, there precipitated 2-(6′-acetamino-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylic acid (284 mg, 0.88 mmole, 88%), mp 229–233° dec (darkens at 210°), showing one main spot in addition to two minor ones on paper chromatography. This preparation did not show any light when tested with ATP, luciferase, Mg^{2+}, and oxygen in the usual way. The material had a yellow color.

Anal. Calcd for $C_{13}H_{11}N_3O_3S_2$: C, 48.58; H, 3.45; N, 13.08; S, 19.96. Found: C, 48.47; H, 3.51; N, 13.14; S, 19.78.

Trifluoroacetylaminoluciferin (IVc). A. 2-Chloro-6-trifluoroacetylaminobenzothiazole (IIc). 2-Chloro-6-aminobenzothiazole (IIa) (1.0 g, 5.42 mmoles) in 300 ml of dry ether was treated at 0° with 2 ml of trifluoroacetic anhydride. The mixture was allowed to warm up to room temperature and to stand for 1 hr. The mixture was evaporated and the residue was recrystallized from 500 ml of isooctane to yield 1.43 g (5.10 mmoles, 94%) of the amide, mp 164–168°. The product was treated with activated charcoal and recrystallized again from octane to give the analytical sample, mp 165–168°.

Anal. Calcd for $C_9H_4ClF_3N_2OS$: C, 38.52; H, 1.44; N, 9.98; S, 11.43. Found: C, 38.57; H, 1.51; N, 10.08; S, 11.99.

B. 2-Cyano-6-trifluoroacetylaminobenzothiazole (IIIc). Potassium cyanide (304 mg, 4.67 mmoles) was dissolved in 60 ml of dimethyl sulfoxide at 150° in a flask equipped with a drying tube. 2-Chloro-6-trifluoroacetylaminobenzothiazole (1.055 g, 3.76 mmoles) was added and the mixture was heated to 150° for 5 hr with stirring (or until the ultraviolet spectrum of an aliquot in ethanol and HCl showed no further growth at 312 mμ). The mixture was cooled and then poured into a mixture of 350 ml of 0.1 *N* KH_2PO_4 and 350 ml of ether. The ether solution was separated and dried; silica gel was placed on the top of a prepared column of silica gel in petroleum ether. The fractions were evaporated, and all fractions giving material melting in the range between 182 and 185° were combined to give 530 mg (1.96 mmoles, 52%) of 2-cyano-6-trifluoroacetylaminobenzothiazole. Recrystallization from isooctane and chloroform yielded crystals melting at 189–191°.

Anal. Calcd for $C_{10}H_4F_3N_3OS$: C, 44.28; H, 1.49; N, 15.49; S, 11.82. Found: C, 44.13; H, 1.59; N, 15.48; S, 11.96.

C. 2-(6′-Trifluoroacetylamino-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylic Acid (IVc). D,L-Cystine (280 mg, 1.16 mmoles) was reduced with sodium in liquid ammonia under dry nitrogen. The excess sodium was destroyed by the addition of solid ammonium chloride and the ammonia was evaporated in a stream of nitrogen. The dry residue was dissolved in water (10 ml, oxygen free) and the pH was adjusted to about 7.5. Methanol (25 ml, boiled under nitrogen) was added. A solution of 2-cyano-6-trifluoroacetylaminobenzothiazole (701 mg, 2.58 mmoles) in methanol (20 ml, oxygen free) was added to the reaction mixture; the reaction was then allowed to proceed for 75 min in dim light. Water was added (100 ml) and the pH was readjusted to 7.6 by the addition of 2 drops of 10% sodium hydroxide. The mixture was extracted with ethyl acetate (100 ml) and the water layer was acidified with hydrochloric acid to a pH of 1. The resulting precipitate was collected after standing overnight in the refrigerator, and it was washed with water. The material was dried at 65° at reduced pressure to yield 652 mg (1.74 mmoles, 74.5%) of yellowish trifluoroacetylaminoluciferin. For analysis, part of this material

was dissolved in hot acetone, and cyclohexane was added to the point at which the first cloudiness developed. On standing, crystals formed, mp 209–211° dec.

Anal. Calcd for $C_{18}H_8F_3N_3O_3S_2$: C, 41.60; H, 2.15; F, 15.18; N, 11.20; S, 17.08. Found: C, 42.05; H, 2.10; F, 16.7; N, 11.96; S, 16.20.

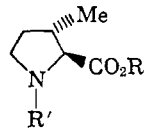
The Stereochemistry of 3-Methylproline

A. B. Mauger, F. Irreverre, and B. Witkop

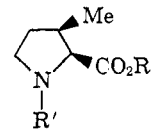
Contribution from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014.
Received December 27, 1965

Abstract: The known product (VIII) of Michael condensation of crotonaldehyde with diethyl acetamidomalonate can be readily dehydrated to the enamide (VII), or converted to the related N-acetyl-4,5-dehydro-3-methylproline ethyl ester, which was separated into *cis* and *trans* forms. These diastereoisomers were key intermediates in the correlation of *cis*- and *trans*-3-methylprolines with alloisoleucine and isoleucine. For example, the *cis* enamide XI was converted, by hydrogenation and hydrolysis, to *cis*-3-methylproline (II), while reaction of XI with ethyl mercaptan gave a mercaptal (XV), which was desulfurized to N-acetylalloisoleucine ethyl ester (XVII). These correlations confirmed the stereochemical assignments based upon preferential saponification, in which isomeric mixtures of N-protected 3-methylproline esters gave a *cis* ester and a *trans* acid; the latter procedure was also useful for separation of the isomers. The nmr spectra of *cis*- and *trans*-3-methylprolines and their derivatives are discussed. By comparison with its published spectrum it is confirmed that the 3-methylproline in bottromycin A is *cis*.

The compound 3-methylproline has been shown to have a potent inhibitory effect upon the biosynthesis of actinomycin in *Streptomyces antibioticus*.[1] In order to study separately the effects of the stereoisomers of 3-methylproline, its separation into *cis* and *trans* forms was undertaken. A convenient synthesis of 3-methylproline was recently described;[2] the diastereoisomeric racemates were separated by crystallization, but not identified. The nmr coupling constants J_{23} of the N-*p*-toluenesulfonyl derivatives[3] were 4.6 and 7.2 cps and the melting points of the free amino acids were 218–219° and 210–211°, respectively. We now present evidence that the former isomer is *trans* (I) and the latter *cis* (II).[4]



I, R = R′ = H
IV, R = H; R′ = Tos
VI, R = Me; R′ = Tos
XIV, R = Me; R′ = Ac



II, R = R′ = H
III, R = Me; R′ = Tos
V, R = H; R′ = Tos
XIII, R = Et; R′ = Ac

Separation of 3-methylproline into racemic *cis* and *trans* forms by ion-exchange chromatography was effective, but for large-scale separations, preferential saponification was more convenient. Saponification of N-*p*-toluenesulfonyl-3-methylproline methyl ester in methanolic sodium hydroxide was continued until one isomer was 96% hydrolyzed, while the other remained 95% intact. Glpc was used to follow the reaction and the acid and ester were separated by extraction. The more resistant ester was the sterically hindered *cis* form (III), while the acid fraction consisted principally of the *trans* form (IV). Hydrolysis of III to V was effected with a hot mixture of hydrochloric and acetic acids, and IV and V converted to the free amino acids by conventional means.[2]

The coupling constant J_{23} was greater for the *cis* derivatives III and V than for the *trans* derivatives IV and VI, and the same relationship held for all the derivatives of 3-methylproline which have been prepared (Table I). By analogy with the case of 3-hydroxyproline,[5,6] this alone gave a tentative basis for stereochemical assignments. However, comparison of J_{23} for the *free* amino acids was difficult, because recognition of the 2-proton signal in *trans*-3-methylproline was obscured by the signals for the 5 protons. In *cis*-3-methylproline, the 2 proton gives a doublet at lower field. This problem was resolved by preparing 4,5-dideuterio-*trans*-3-methylproline as described below. Examination of its nmr spectrum and that of the unlabeled substance shows a J_{23} comparable in magnitude with that of the *cis* compound (Table I). This could be explained by a degree of flexibility of the ring in the free amino acids which is higher than in their derivatives carrying substituents on nitrogen. Rapid fluctuation of dihedral angle between the 2 and 3 protons in both the *cis* and *trans* isomers would give observed coupling constants which are an "average" of maximum and minimum values. The two spectra show different chemical shifts for both the 2 proton and 3-methyl protons, and this can be accounted for in terms of steric hindrance to rotation of the carboxyl (or carboxylate anion) function in the *cis* isomer. Space-

(1) T. Yoshida, A. B. Mauger, B. Witkop, and E. Katz, 148th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1964, Abstracts, p 40C; also in press.
(2) D. A. Cox, A. W. Johnson, and A. B. Mauger, *J. Chem. Soc.*, 5024 (1964).
(3) The coupling constants J_{23} given in ref 4 refer to the N-*p*-toluenesulfonyl derivatives, not the free amino acids as stated.
(4) A. B. Mauger, F. Irreverre, and B. Witkop, *J. Am. Chem. Soc.*, 87, 4975 (1965).
(5) F. Irreverre, K. Morita, A. V. Robertson, and B. Witkop, *ibid.*, 86, 8293 (1964).
(6) J. Blake, C. D. Willson, and H. Rapoport, *ibid.*, 86, 5293 (1964).