Bioconversion of Arteannuin B to Artemisinin

M. S. R. Nair, and D. V. Basile

J. Nat. Prod., **1993**, 56 (9), 1559-1566• DOI:
10.1021/np50099a015 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 9, pp. 1559-1566, September 1993

BIOCONVERSION OF ARTEANNUIN B TO ARTEMISININ[1]

M.S.R. NAIR* and D.V. BASILE

Department of Biological Sciences, H.H. Lehman College of CUNY, The Bronx, New York, 10468

ABSTRACT.—Arteannuin B, which co-occurs with artemisinin, the potent antimalarial principle of the Chinese medicinal herb *Artemisia annua* (Asteraceae), has been converted to the latter using crude and semi-purified cell-free extracts of the leaf homogenates of the plant. Detection procedures to quantitate this bioconversion, including one that is novel which uses gc-ms, are detailed.

Extracts of the herb qinghao, *Artemisia annua* L. (Asteraceae), have been used in the Chinese traditional medicine for centuries to treat the chills and fevers of malaria (1). The active principle, artemisinin (Qinghaosu, QHS) was isolated in pure form in 1972, and its structure was determined in 1979 (2–5). Artemisinin and some of its more lipophilic (injectable) and hydrophilic (po) derivatives (6–10) have been used to treat tens of thousands of malarial patients in the People's Republic of China. These derivatives, which can only be obtained from the parent compound, were prepared to enhance drug delivery and are up to 8–10 times as potent as artemisinin itself. Since the clinical use of artemisinin and its derivatives was initiated in the seventies, no significant adverse side effects have been observed, even in cases complicated by cardiac, renal, and hepatic disorders or by pregnancy (11,12). Recently a few instances of teratogenicity and abortifacient effects of this drug have been reported (13). Despite this statistically insignificant observation, the benefit to an overwhelming majority of the patients and their high therapeutic indices make artemisinin and its derivatives, by far, the safest and most desirable group of drugs for the treatment of chloroquine-resistant *Falciparum malaria*, especially the cerebral infections (14). Several total syntheses of artemisinin have been reported (15–18) as well as a recent three-step synthesis starting from artemisinic acid (19), but the cost of the synthesized drug is too high to be affordable by the patients where malaria is highly endemic. The *A. annua* plant itself is, therefore, the only practical source of this valuable drug. Unfortunately, this annual is adapted to growing in the temperate zones of Eurasia and the Americas, and the strains presently available have not been cultivated in the tropic and subtropic regions where they are most needed.

The present work was undertaken to find an alternate source of this drug by the bioconversion of less complex, and thus more easily accessible, precursors, employing cell-free enzymes derived from the plant itself or from its cell-suspension cultures (20). To facilitate the quantitation of the conversion, a novel procedure using gc-cims, especially adapted for the analyses of "artemisinoids," was developed. Results reported here pertain to the conversion of arteannuin B, an inactive constituent of *A. annua*, that is more readily available than artemisinin. The amount of arteannuin B present in the leaves is dependent on the stage of development of the plant when it is harvested. For example, when the plants are harvested at or just after the first inception of flower buds, the amount of arteannuin B represents only 40% of that of artemisinin. However, when the plants were harvested well before flower initiation (e.g., 6–7 weeks after germination), the level of arteannuin B is as much as 2.9 times of that of artemisinin.

An added attraction in using arteannuin B as a potential starting material for cost-effective production of artemisinin is that a number of successful syntheses of arteannuin

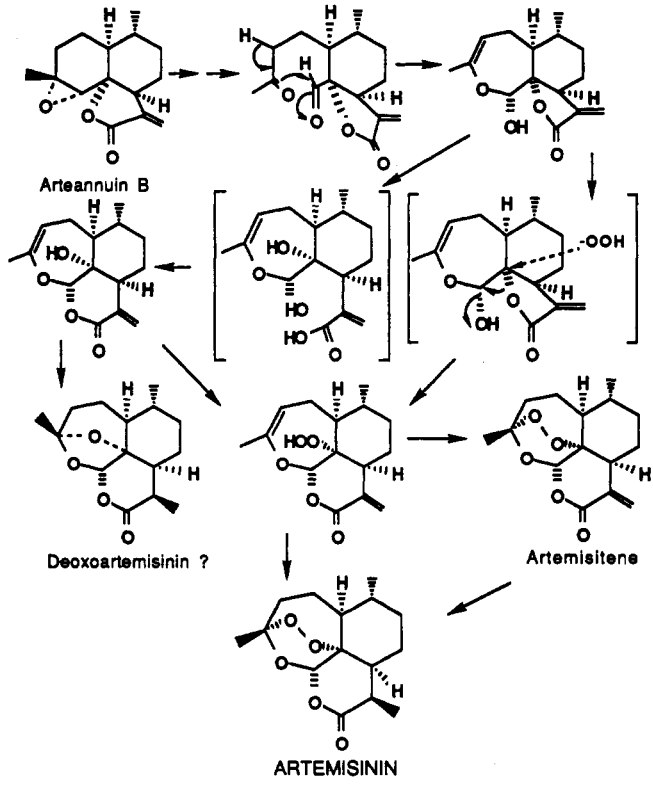
[1]Parts of this report were presented at the International Congress of Natural Products, University of North Carolina, Chapel Hill, NC, July 7–12, 1985.

B, some in high yields and in a few steps (21–23), have been reported. All efforts to convert arteannuin B to the effective drug by enzymatic or microbial transformations have been of no avail. Very recently Lansbury and Novak (24) prepared an intermediate in two steps from arteannuin B (43% yield), which could be converted to artemisinin in "four to five" steps in an overall yield (based on arteannuin B) of about 10%. In 1984 we were successful in effecting this conversion using crude cell-free extracts of the leaves of the plant, and we proposed a hypothetical pathway for this conversion (25), represented in Scheme 1, which also gives the structures of arteannuin B and artemisinin.

After we first reported this bioconversion (25), El-Feraly *et al.* (26) suggested that artemisinic acid is a possible precursor of artemisinin. Wang *et al.* (27) converted artemisinic acid ^{3}H-labeled at C-15 (exocyclic methylene) to both arteannuin B and artemisinin. But their results did not show whether artemisinic acid is converted to artemisinin via the intermediacy of arteannuin B. We recently reported (28) results which clearly indicate that arteannuin B is an intermediate in the bioconversion of artemisinic acid to artemisinin. Staba's group (29,30) converted isopentenyl pyrophosphate to both arteannuin B and artemisinin. Akhila and co-workers (31,32) used [^{13}C,^{1}H]-doubly labeled mevalonic acid to study the biogenesis of artemisinin and concluded that arteannuin B is the immediate precursor of artemisinin. Aside from our studies, which used cell-free extracts, all the other conversions used either cell cultures or a wick method to introduce the precursors.

RESULTS AND DISCUSSION

Here we report results from dozens of experiments using cell-free leaf homogenates



SCHEME 1. Hypothetical bioconversion pathway of arteannuin B to artemisinin.

of *A. annua* plants of different origins grown in controlled and wild conditions. Parameters such as the buffers, extraction protocols, and incubation conditions were systematically changed. We used crude cell-free extracts as well as extracts semi-purified by ultrafiltration. These experiments were conducted during a span of several seasons, and the seasonal variations of the weather and hence growth rates of the plants also affected the results. The plants were not axenically grown and the results from one season had to be discarded because the plants were infested by insects.

The first sets of experiments used plants collected from the wild in Virginia and maintained at the greenhouse and grounds of Walter Reed Army Institute of Research (WRAIR). At the first inception of the buds, the cell-free leaf extracts to which arteannuin B was added gave after incubation up to four times as much of artemisinin as the control (Figure 1). During subsequent weeks artemisinin content in the experiment was the same as that in the control, within experimental error. As it has been observed that the artemisinin content in the plant declines steadily once the flowering starts, it is reasonable to assume that the enzyme(s) responsible for this conversion is absent at this particular growth-stage of the plant. Artemisinin was detected and quantitated by liquid chromatography-electrochemical detection (lc-ec), by courtesy of WRAIR, whenever possible. But since quantitation using uv absorption at 292 nm of the alkali transformation product of artemisinin gave comparable results (within experimental error of <4% either way), only the latter method was used in the experiments when the WRAIR facility was not available. To further confirm the validity of the uv assay, a standard solution of artemisinin was analyzed by both methods and the results were found to be identical.

In the seasons of 1989 and 1990 plants collected on or near the Lehman College Campus in The Bronx, New York, were used for these studies. Leaves from field-collected plants and from those potted and kept at controlled conditions in the experimental greenhouse were employed. The Bronx variety when sucrose-TRIS buffer was used for extraction gave a much lesser degree of conversion of arteannuin B than the Virginia

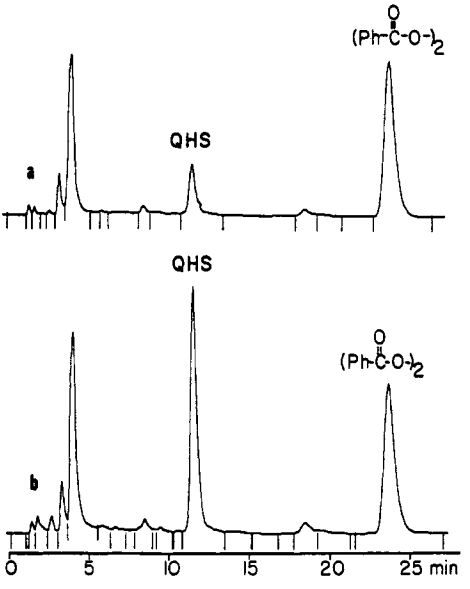


FIGURE 1. Bioconversion of arteannuin B to artemisinin, lc-ec traces: (a) Control, (b) Experiment: Control + arteannuin B.

variety (25–45%). To determine whether this anomaly was due to the change in buffers, four different buffers, Sucrose-TRIS containing cysteine, TRIS-Dithioerythritol (DTE), HEPES-Dithiothreitol (DTT), and Barbitol-DTT-Ethylenediaminetetraacetic acid (EDTA) were used to extract the leaf homogenates. The last three gave comparable results of 28 ± 1 μg/g for control and 41 ± 1 μg/g for the experiment, a 45 ± 1% increase. However, the sucrose-TRIS buffer gave only 19 μg/g for the control and 28 μg/g for the experiment but about the same (45%) increase in the formation of artemisinin. This experiment was done during the last week of August 1992 when the flowering had already started. These results showed that sucrose-TRIS buffer is not suitable to extract the maximum amount of the metabolites, and they also showed that the Bronx strain is inferior to the Virginia strain since the latter at the same growth stage gave over 80% increase in artemisinin content, 53.4 μg/g for the experiment compared to 29.5 μg/g for the control.

During the 1990 season in New York, the weather in the spring was cold and by mid-June the plants were only ca. 6 weeks old. Extraction in this case was done using HEPES buffer containing DTT so that incubations could be conducted at variable temperatures as this buffer undergoes pH changes minimally with temperature. In this experiment, both lc-ec and uv detection at 292 nm of the alkali transformation product gave exactly the same amount (90 μg/g) of artemisinin in the experiment and control. We suspected that arteannuin B present in the crude cell-free extract got converted to artemisinin, and addition of exogenous arteannuin B had no effect. This hypothesis was tested by quenching the control cell-free enzymes immediately after their preparation. After incubation, the ratio of artemisinin content in the experiment (90 μg/g) to that in quenched control (62 μg/g) was about 45%. This in itself does not prove that arteannuin B is the precursor which was transformed and not one of the other components of the mixture. To verify this point, we ultrafiltered the cell-free extract to remove all molecules of less than 10,000 in mol wt. A control and an experiment with arteannuin B (150 μg) were incubated at 37° with ATP and Mg^{2+} and Mn^{2+} ions. The control did not have any artemisinin, and the experiment showed 85 μg/g of artemisinin. This proved beyond doubt that arteannuin B is a genuine precursor of artemisinin and also tended to indicate that when the artemisinin content in the mixture reaches a critical level compared to the amount of enzymes present, the bioconversion stops. The gc-cims showed that the mixture still contained arteannuin B, but the amount of artemisinin produced did account for only 80% of arteannuin B used up.

For the detection and quantitation of artemisinin, several methods have been employed involving uv spectroscopic, lc-ec, and tlc assays. Tlc followed by derivatization and uv scanning, though attractive in its simplicity, lacks the desired accuracy and reliability. Hplc using ec detection (33), and using uv detection after modification by treatment with NaOH (34) are highly reliable methods for the determination of artemisinin and we, as described earlier, used both of these procedures. Artemisinic acid, making use of its carboxyl function, can conceivably be detected by uv-vis spectra by suitable derivatization, even though it has, to our knowledge, never been done. Arteannuin B can not be detected or quantitated by any of these procedures. Hplc using uv detection at 220 nm has been employed for the determination of the retention times and purity of artemisinin, arteannuin B, and artemisinic acid, isolated in pure form employing flash cc in the first two cases and solubility in $NaHCO_3$ in the case of artemisinic acid (35). However, since they do not have an absorption maximum at this wavelength and since other compounds present in the crude extracts interfere in this region, this procedure is of little use in analyzing crude samples. Since our experiments needed a means to determine the concentration of arteannuin B required for optimum

bioconversion, we had to design a new assay procedure (28). Mass spectral studies (ei and ci modes) (36) showed thermal decomposition as well as easy fragmentation of the molecular ion. However, thermolytic studies on artemisinin report (37) that neat artemisinin is stable up to 50° above its melting point (156–157°) for 2.5 min, and extensive changes are observed only after 10 min at 190°. Therefore we decided to analyze an equimolar mixture of artemisinic acid, arteannuin B, and artemisinin by gc-ms in ci mode using NH_3 as reagent gas. The three compounds gave distinct peaks, and under the same conditions, the mixture gave reproducible retention times and same ratio of the peaks corresponding to $[M+NH_4]^+$ ions at *m/z* 252, 266, and 300 for the three compounds. All the three compounds had retention times of less than 6 min, and the oven temperature never reached above 190° before the compounds were eluted from the column. However, extensive decomposition of artemisinic acid and artemisinin occurred, and the ratio observed for the equimolar mixture was 1:11.4:2.4 in the order of decreasing mol wt. Whereas artemisinic acid gave copious amounts of $[M+H]^+$ ion, arteannuin B and artemisinin gave only $[M+NH_4]^+$ species. The normalized gc-ms curves (Figure 2) give the ratio of the precursor to the drug in the control, the quenched

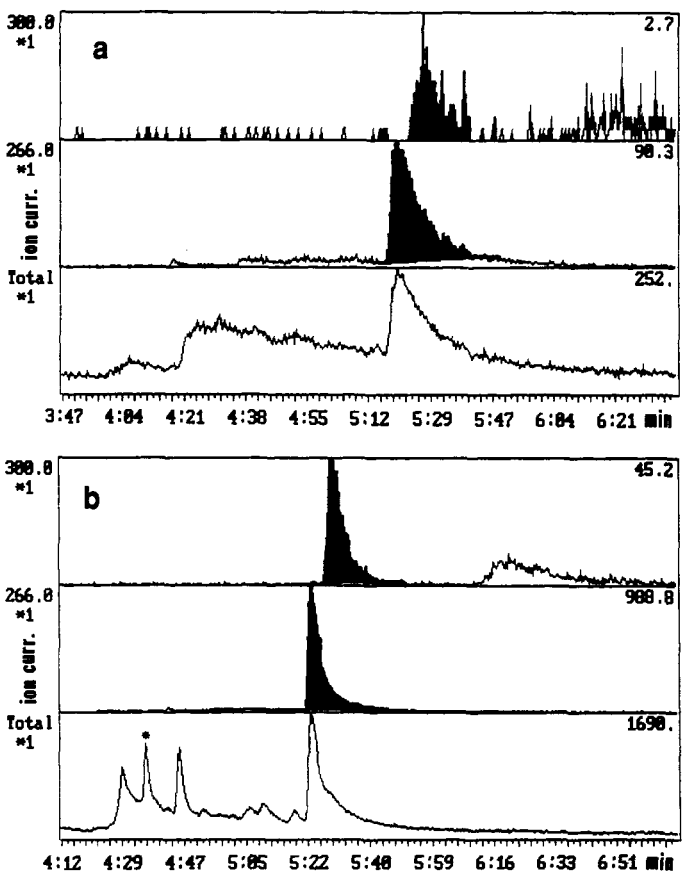


FIGURE 2. Normalized gc-cims (NH_3) curves showing peaks for artemisinin at *m/z* 300, $[M+NH_4]^+$ and for arteannuin B at *m/z* 266, $[M+NH_4]^+$: (a) Cell-free extract quenched before incubation. Normalized ion current curves show the ratio of arteannuin B to artemisinin is 2.9:1. (b) Cell-free extract quenched after incubation. Normalized ion current curves show that the ratio of arteannuin B to artemisinin has been reduced to 1.9:1. A slight overlap at *m/z* 266 is due to the $[M-O]^+$ ion from artemisinin. Numbers at the top right-hand corners give the integrated values of the ion currents. Artemisinic acid is shown with an asterisk.

cell-free extract of *A. annua* leaves (ca. 6 weeks old), and the experiment using unquenched extract, respectively. From the expected 11.4 times abundance of the arteannuin B peak, it can be seen that these young plants contain 2.9 times arteannuin B than artemisinin, in the quenched extract (Figure 2). This, to our knowledge, is the only procedure reported so far that can identify and quantitate all these three important metabolites of *A. annua* simultaneously. After the completion of our studies (28) and the original submission of this paper, Woerdenbag *et al.* (38) reported using a gc-ms method to identify artemisinoids and to determine their Rt's so that they could be separated by gc.

The results reported above, especially the ones involving plantlets, show that after the concentration of artemisinin reaches a particular level, no more formation of it takes place. Since artemisinin is less polar than arteannuin B and is eluted by hexane-Et_2O (9:1), whereas the arteannuin B is eluted only by hexane-Et_2O (4:1) (21), the former can be periodically removed by flash cc for continuous bioconversion of the latter using immobilized enzymes. In conclusion, origin of the plants, weather, and soil conditions all affect the efficacy of the leaf homogenates to effect the bioconversion. We are now pursuing the same bioconversion using cell-free extracts from suspension cultures, which are less prone to the variables. Furthermore, they have a much shorter life span, and hence can be monitored more effectively.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Hplc was run using an ODS column, Millipore 30 cm×3 mm i.d.; sample volume 1 μl at a flow rate of 1 ml/min and 1800 psi using NH_4OAc buffer-MeCN (55:45) predegassed with He, and artemisinin was detected by ec using a gold-mercury electrode at a potential of −0.8 V. Gc-ms used a capillary column, Supelco SPB-1, 15 m×0.25 mm i.d.; injection block 200°; oven 100–200° at 15°/min and was analyzed by Ribermag R-10-10 MS in the ci(NH_3) mode, scanning at 2 sec per spectrum. Uv absorption was determined using a Varian-634 spectrophotometer. All the amounts of the metabolites are given as μg/g of wet wt of the *A. annua* leaves.

PLANT MATERIAL.—Plants were collected from wild growth in Virginia and were maintained in a greenhouse. These and the plants cultivated in the grounds of WRAIR were used in the preliminary experiments. Specimens are on deposit at the U.S. National Arboretum, Washington DC. During subsequent seasons, field-collected plants of *A. annua* growing in or near our campus were collected and were kept potted in our experimental greenhouse or the leaves were collected fresh from plants growing in the field. These plants were identified by the experts at The New York Botanical Garden, Bronx, NY, and are on deposit there.

BUFFERS.—Four different buffers were used for extraction: TRIS-DTE, Sucrose-TRIS, HEPES-DTT, and Barbitol-DTT-EDTA with their pH's at 25° adjusted to 7.0, 7.5, 7.1, and 7.2, respectively. All of them were 50 mM and the stabilizers were added at 2 mM concentrations.

PREPARATION OF THE CELL-FREE EXTRACTS.—Two methods were employed to determine the most suitable procedure: In a typical experiment the potted plant was kept overnight at 4° or the leaves with stem from the wild were kept in a beaker of water in a 4° coldroom for 24 h. Leaves and tender stems were clipped off (ca. 20 g wet wt) and were homogenized in a tissue grinder using 20 ml of the buffer. The extract was centrifuged at 4° for 1 h at 100,000×*g*. In the other method, at 4°, 10 g of the leaves were homogenized first in a Waring blender using 20 ml of the buffer and further homogenized in a tissue grinder and were centrifuged at 2000×*g*. The supernatant was filtered successively through Whatman No 4 filter and 0.5 μ and 0.25 μ filters (Millipore) using Sweeney units. Both methods were equally effective.

INCUBATION OF ARTEANNUIN B WITH THE CELL-FREE SYSTEM.—Two equal amounts of the cell-free extracts (5 ml) were transferred to stoppered tubes and one was used as control. To both the portions, 0.1 mM ATP and 0.1 mM Mg^{2+} and Mn^{2} sulfates in 1 ml buffer were added. In the experiment, 50–200 μg arteannuin B in 1 ml buffer was added and in the control 1 ml of buffer alone was introduced. The tubes were stoppered and incubated at 30° for 3 h in a shaking water bath, at the end of which time the enzymes were quenched by adding 1 ml of EtOH-CHC_3 (1:1). In the experiments involving young plants, one part, the control, was quenched immediately after the preparation of the cell-free homogenate.

When HEPES buffer, which is less prone to variations of pH with temperature, was used, the

incubation was conducted at 30° for 2 h followed by leaving the mixture overnight at 4° for 24 h, or at 4° for 24 h followed by 2 h at 30°. No significant difference in the degree of bioconversion was observed.

ULTRAFILTRATION OF THE CELL-FREE EXTRACTS.—Centriprep-10 Concentrator (Amicon) with 10,000 D cut-off was used at 2500×*g* to remove low mol wt secondary metabolites from the cell-free extracts of younger plants which contained large amounts of arteannuin B and artemisinic acid, so that the effect of the exogenous precursor could be studied without interference from the natural precursors.

EXTRACTION OF ARTEMISININ.—The incubation mixture was diluted with an equal amount of H_2O (7 ml) and extracted with 2×10 ml hexane. Hexane was removed, and the residue was taken up in 5 ml of MeCN.

DETECTION AND QUANTITATION OF ARTEMISININ.—*Hplc with ec detection.*—The ec detector was calibrated using a stock solution of pure artemisinin, and benzoyl or trityl peroxide as internal standard. The MeCN-soluble portions of the hexane extracts of the quenched incubation mixtures were analyzed, after diluting so that 1 ml represented 1 g of the plant material, following the general procedure described by Acton *et al.* (33). The Virginia variety of the plant showed a maximum conversion, up to four times the amount of artemisinin as the control, when 200 μg of the precursor was employed and when leaves of plants which had just begun to form flower buds were used (Figure 1). Bronx plants showed 25 to 45% increase when 50 μg of arteannuin B was used. Increase of the amount of 100 μg showed no difference.

Uv detection by modification of artemisinin by alkali.—On treatment with NaOH, artemisinin undergoes the opening of the lactone ring to give an intermediate in which interlocking functionalities are set free; subsequent changes culminate in the formation of a species which absorbs at 292 nm (34). The extract of the incubation mixture representing 1 g of the wet plant material in MeOH (2 ml) was mixed with aqueous NaOH (1 N, 2 ml). The mixture was heated at 50° for 30 min. The uv absorption at 292 nm of the alkaline solution was measured. When needed (as was usually the case) the mixture was diluted with MeOH to obtain an OD of ca. 0.7 and suitable adjustments were made in the reference solution by dilution of the 2% NaOH solution in 50% MeOH by adding the required amount of MeOH. An OD of 1.0 represents 17.1 μg of artemisinin (determined experimentally).

Gc-cims (NH_3) analysis.—The MeCN-soluble portions of the hexane extracts of the control and experiment were taken to dryness, and the residue was dissolved in $CHCl_3$ (1 ml solution representing 1 g of the plant material). The solution (1–4 μl, depending on the concentration of artemisinin and arteannuin B present) was injected in the gc-cims using NH_3 as reagent gas. Arteannuin B appeared at 5.18 and artemisinin at 5.27 min. The peaks were normalized and integrated. These peaks attributed to arteannuin B and artemisinin were then analyzed by gc-eims, and the ms was the same as those of the pure compounds taken under the same conditions.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of the Division of Experimental Therapeutics of Walter Reed Army Institute of Research where the preliminary work was initiated with partial support from NRC. We are especially thankful to Dr. N. Acton and the late Dr. D.L. Klayman of WRAIR who provided authentic samples as well as the lc-ec curves. We thank Ms V. Parmakovic of Columbia University for the mass spectra. This work was supported in part by a grant 5RO1-Al28975 from the National Institute of Allergy and Infectious Diseases of NIH.

LITERATURE CITED

1. D.L. Klayman, *Science,* **228**, 1049 (1985).
2. Anonymous, Co-ordinating Group for Research on the Structure of Qing Hao Su, *K'o Hsueh T'ung Pao,* **22**, 142 (1977); *Chem. Abstr.,* **97**, 98788 (1977).
3. J. Liu, M. Ni, J. Fran, T. Yu, and Z.Wu, *Acta Chem. Sin.,* **37**, 129 (1979).
4. H.M. Liu, K.I. Li, and W.O. Wo, *Yao Hsueh T'ung Pao,* **15**, 39 (1980); *Chem. Abstr.,* **95**, 12030e (1981).
5. Qinghaosu Res. Group, *Sci. Sin (Engl. Ed.),* **23**, 380 (1980).
6. G.Q. Li, X. Gao, K. Arnold, H. Jian, and L. Fu, *J. Tradit. Chin. Med.,* **2**, 125 (1982).
7. Anonymous, China Cooperative Res. Group, *J. Tradit. Chin. Med.,* **2**, 9 (1982).
8. A.J. Lin, D.L. Klayman, and W.K. Milhous, U.S. Patent Appl., U.S. 87,365, May 15, 1989; *Chem. Abstr.,* **110**, 193144w (1989).
9. A.J. Lin and D.L. Klayman, *J. Med. Chem.,* **32**, 1249 (1989).
10. A. Brossi, B. Venugopalan, L. Dominguez-Garpe, H.J.C. Yeh, J.L. Flippen-Anderson, P. Buchs, X.D. Luo, W.K. Milhous, and W. Peters, *J. Med. Chem.,* **31**, 645 (1988).

11. Anonymous, China Cooperative Research Group, *Chin. Med. J.*, **92**, 811 (1985).
12. X. Niu, L. Ho, Z. Ren, and Z. Song, *Eur. J. Drug Metab. Pharmacokin.*, **10**, 55 (1985).
13. Z.L. Li, *Chung Yao T'ung Pao*, **13**, 42 (1989); *Index Medicus*, **30**, 2114 (1989).
14. G.Li, X. Guo, R. Jin, Z. Wang, H. Jian, and Z. Li, in: "Abstracts." International Congress for Tropical Medicine and Malaria, Calgary, Canada, Sept. 1984.
15. G. Schmid and W. Hofheinz, *J. Am. Chem. Soc.*, **105**, 624 (1983).
16. W. Zhou, *Pure Appl. Chem.*, **58**, 817 (1986).
17. X.X. Xu, J. Zhou, D.Z. Huang, and W.S. Shou, *Tetrahedron*, **42**, 1819 (1986).
18. T. Ravindranathan, M.A. Kumar, R.B. Menon, and S.V. Hiremath, *Tetrahedron Lett.*, **31**, 68 (1990).
19. R.J. Roth and N. Acton, *J. Nat. Prod.*, **52**, 1183 (1989).
20. M.S.R. Nair, N. Acton, D.L. Klayman, K. Kendrick, D.V. Basile, and S. Mante, *J. Nat. Prod.*, **49**, 504 (1986).
21. M. Jung, Y. Yao, H.N. ElSohly, and J.D. McChesney, *J. Nat. Prod.*, **50**, 972 (1987).
22. X. Xu, J. Zhu, and W. Zhou, *Xuaxue Xuebao*, **43**, 48 (1985).
23. P.T. Lansbury and C.A. Mojica, *Tetrahedron Lett.*, **27**, 3967 (1986).
24. P.T. Lansbury and D.M. Novak, *Tetrahedron Lett.*, **33**, 1029 (1992).
25. M.S.R. Nair, N. Acton, D.L. Klayman, K. Kendrick, and S. Mante, in: "Abstracts," (Abstr. 75, cf. refs. 20, 30) International Congress on Natural Products, Chapel Hill, NC, July 7–12, 1985.
26. F.S. El-Feraly, I.A. Al-Meshal, M.A. Al-Zahya, and M.S. Hifnawy, *Phytochemistry*, **25**, 2777 (1986).
27. Y. Wang, Z. Xia, F. Zhou, Y. Wu, and J. Huang, *Xuaxue Xuebao*, **46**, 1152 (1988); *Chem. Abstr.*, **110**, 111841j (1988).
28. M.S.R. Nair and D.V. Basile, *Indian J. Chem.*, **31B**, 880 (1992).
29. J. Kudakasseril, L. Lam, and E.J. Staba, *Planta Med.*, 280 (1987).
30. B.C. Martinez and E.J. Staba, *Adv. Cell Cult.*, **6**, 69 (1988), and references cited therein.
31. A. Akhila, R.S. Thakur, and S. Popli, *Phytochemistry*, **26**, 1927 (1987).
32. A. Akhila, K. Rani, and R.S. Thakur, *Phytochemistry*, **29**, 2129 (1990).
33. N. Acton, D.L. Klayman, and I.J. Rollman, *Planta Med.*, 445 (1985).
34. S.S. Zhao and M.Y. Zeng, *Planta Med.*, 233 (1985).
35. N. Acton and R. Roth, *J. Chem. Ed.*, **66**, 349 (1989).
36. K.P. Madhusudanan, R.A. Vishwakarma, S. Balachandran, and S.P. Popli, *Indian J. Chem.*, **28B**, 751 (1989).
37. A.J. Lin, D.L. Klayman, J.M. Hoch, J.V. Silverton, and C.F. George, *J. Org. Chem.*, **50**, 4504 (1985).
38. H.J. Woerdenbag, N. Pras, R. Bos, J.F. Visser, H. Hendriks, and T.M. Malingre, *Phytochem. Anal.*, **2**, 215 (1991).

Received 11 March 1993