Diverted total synthesis: Preparation of a focused library of latrunculin analogues and evaluation of their actin-binding properties

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Two largely catalysis-based and highly convergent total syntheses of latrunculin A (1) and B (2) were diverted to the preparation of a focused library of analogues of these potent actin-binding macrolides that enjoy widespread use in chemical biology. Because the chosen route allows for structural variations of all characteristic parts of the natural leads, it was possible to map the previously largely unknown structure/activity profile of this class of bioactive natural products. This led to the discovery that the removal of the methyl branches decorating the macrocycle in 2 engenders a significant increase in potency, while streamlining the synthesis to a considerable extent. Moreover, compelling evidence is provided that the conspicuous 2-thiazolidinone ring present in all naturally occurring latrunculins may be an optimal but not an essential structural motif for actin binding because it can be replaced by an oxazolidinone moiety with only slight loss in efficacy. Likewise, the inversion of the absolute configuration of the chiral center at C.16 is well accommodated. From the purely chemical perspective, this investigation attests to the maturity of alkyne metathesis, a method that has received attention as efficient means for the formation of macrocycles only recently.

alkyne metathesis | natural products

Although colonizing the densely populated coral reefs of the Red Sea, the sponge *Negombata magnifica* (formerly *Latrunculia magnifica*) is conspicuously free from predation in its natural habitat because of an efficient chemical defense mechanism (1). Molestation or squeezing lead to the secretion of a reddish fluid causing fish poisoning and death within minutes. Bioassay-guided fractionation of this fluid led to the discovery of the latrunculins, a family of 14- and 16-membered lactones and the first natural products embodying a 2-thiazolidinone moiety (Fig. 1) (2–6). These macrolides were later also found in taxonomically unrelated organisms from different locations, thus raising questions as to the actual producer of these intriguing secondary metabolites (7–12).§

In addition to the pronounced ichthyotoxic properties of **1**–**6**, the latrunculins were found to be cytotoxic and antivirally active. Most notable, however, is their potent and selective effect on the actin cytoskeleton without disrupting the microtubular system (13). Actin not only determines the shape and mechanical properties of eukaryotic cells but is also responsible for cell motility processes as fundamental as exo- and endocytosis. Our present knowledge about these biological roles of actin derives to a large extent from a "chemical genetics" approach¶ using probe molecules able to dissect this highly sophisticated and inherently dynamic cellular structure (14, 15). The latrunculins played a prominent role in this regard, not least because of their striking selectivity, their surprisingly rapid onset of action, and their remarkable potency that exceeds that of the cytochalasins by 1–2 orders of magnitude (16). Incubation of nonmuscular cells with **1** or **2** almost immediately triggers dramatic morphological changes. Despite the loss of their usual shapes, the resulting deformed cells continue to grow and metabolize (16–20). Furthermore, the latrunculins alter actin-mediated adhesive interactions in tissue, inhibit fertilization and early development of sea urchin eggs or mouse oocytes, inhibit force development in muscles, disturb microfilament-mediated processes in meiosis, and affect protein kinase C signaling pathways (14–20). Even an actin-dependent checkpoint in mitosis has been discovered recently, which seems to be evolutionary highly conserved (21).

At the molecular level, the latrunculins form 1:1 complexes with actin monomers (globular actin, G-actin) that are incapable of polymerizing to the intact protein filament network (fibrous actin, F-actin). The binding site of **1** has been located by x-ray crystallography in the vicinity of the nucleotide binding cleft of the protein (Fig. 2) (22, 23).

Despite these truly remarkable physiological properties of the latrunculins and their widespread use as biochemical tools, the present understanding of the structure/activity relationship (SAR) of these exquisite actin binders is fairly limited (24–26). While derivatizations of the natural products showed that a free hemiacetal moiety at C.17 (Lat-A numbering) and an unprotected N-atom of the thiazolidinone ring are pivotal for eliciting a biological response,‖ a more detailed mapping of the SAR is warranted. The recently solved structure of the actin–latrunculin A complex may provide guidance in this regard, because it reveals the essential features of the hydrogen-bonding network between the protein and the macrolide ligand, engaging every polar atom in **1**, except the C.15 ester oxygen (Fig. 2) (22). To gain further insights, we launched a synthesis program with the aim of opening access not only to the naturally occurring latrunculins but also to various analogues for biological testing. Thereby, our focus was on the role of individual heteroatoms and the macrocyclic ring, the effect of which is difficult to address by post facto modifications of the natural products.

Materials and Methods

Bioassay. Murine NIH/3T3 fibroblasts (CRL-1658 from American Type Culture Collection) were cultured at 37°C and 5% CO_2 in DMEM supplemented with 4 mM L-glutamine, 4.5 g/liter glucose, and 10% bovine calf serum. Cells (2×10^4) were seeded on coverslips in one well of a 24-well plate. After adapting and

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Abbreviation: SAR, structure/activity relationship.

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§This question is even more puzzling in the light of a recent investigation which makes it plausible that latrunculin B is of nonsymbiotic origin (cf. ref. 4).

¶The striking specificity of the latrunculins is reminiscent of genetic knockout experiments that allow one to inactivate a single component of the highly complex subcellular network. Therefore, the expression "chemical genetics" seems particularly appropriate in this context.

‖Although the introduction of simple *N*-alkyl or *N*-benzyl groups results in complete loss of the biological activity, the *N*-CH_2OH group present in latrunculin E is an acceptable modification.

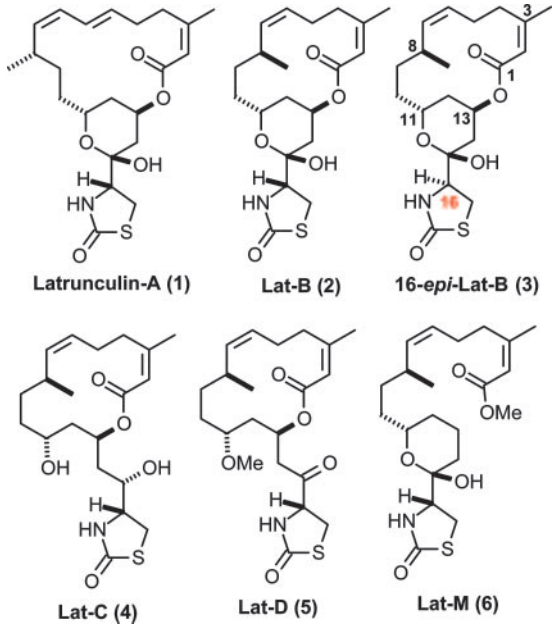
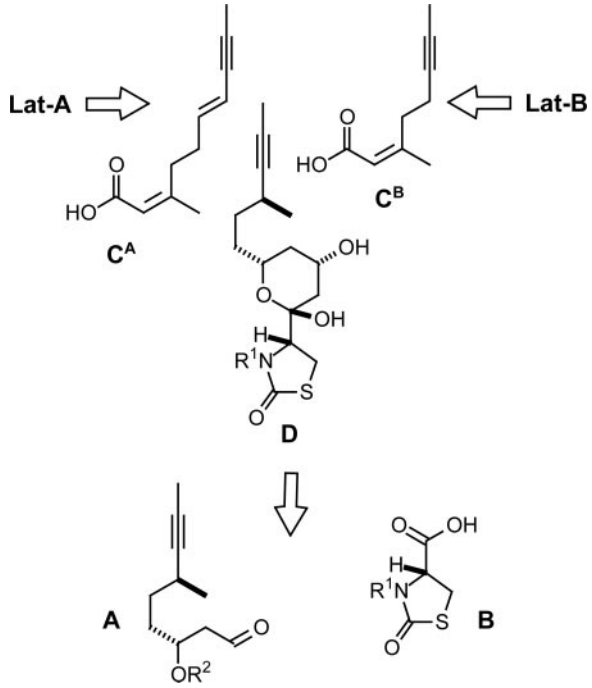


Fig. 1. Structures of some naturally occurring latrunculins.



Scheme 1. Retrosynthetic analysis of Lat-A and B unravels the common building block **D**, which itself derives from two simple synthons **A** and **B**. For the preparation and assembly of these fragments and the completion of the total syntheses by ring-closing alkyne metathesis, see refs. 27 and 28.

attaching overnight, the cells were incubated with 1, 5, or 10 μM of the corresponding latrunculin compound for 18 h. Before and after each fixation or staining step, the cells were washed three times with TPBS (0.2% Tween 20 in PBS). Cells were fixed with 3.7% formalin in PBS. For blocking unspecific epitopes, fixed cells were incubated with 1% powdered milk in PBS. Actin filaments were stained for 1 h with a solution of 77 nM TRITC-labeled phalloidin (P1951, Sigma) in TPBS. Cell nuclei were stained with DAPI [2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; D9542, Sigma]. Cells were visualized and photographed with a Zeiss Axiophot fluorescence microscope.

Representative Procedure for Ring-Closing Alkyne Metathesis. To a degassed solution of the diyne in toluene was added $(t\text{BuO})_3\text{W}{\equiv}\text{CCMe}_3$ (**28**) (10–30 mol %), and the resulting mixture was stirred at 80°C for 2.5 h under Ar. For work-up, the solvent was evaporated, and the residue was purified by flash chromatography to give the corresponding cycloalkyne in analytically pure form in the yields indicated in Scheme 2 or *Supporting Text* (which is published as supporting information on the PNAS web site), respectively.

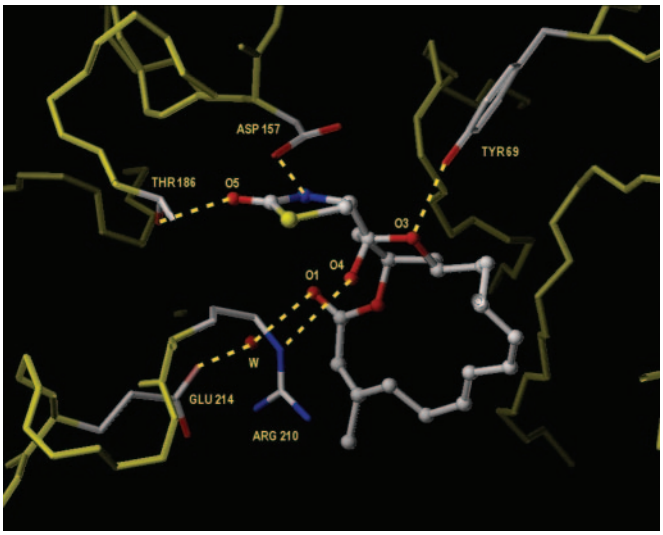


Fig. 2. Pertinent part of the crystal structure of the latrunculin A–actin complex showing the hydrogen bonding network between the ligand and the host (cf. ref. 22).

Representative Procedure for CAN-Induced Global Deprotection: Latrunculin B (2). CAN (31 mg, 0.057 mmol) was added to a vigorously stirred suspension of compound **83** (12 mg, 0.023 mmol) in $\text{MeCN}/\text{H}_2\text{O}$ (2:1, 0.5 ml). After 20 min, the mixture became homogeneous, and stirring was continued for 3 h. The solution was extracted with CH_2Cl_2 (3×), the combined organic layers were dried and evaporated, and the residue was purified by flash chromatography to give **2** as a colorless oil. $[\alpha]_D^{20} = +122°$ ($c = 0.55$, CHCl_3); IR (ATR) 3,328, 2,952, 1,678, 1,278, 1,092, 1,057 cm^{-1}; ^{1}H NMR (400 MHz, CDCl_3) δ 0.95 (d, 3H, $J = 6.3$ Hz), 1.07–2.39 (m, 11H), 1.90 (d, 3H, $J = 1.3$ Hz), 2.60–2.80 (m, 2H), 3.39 (dd, 1H, $J = 6.3, 11.6$ Hz), 3.47 (dd, 1H, $J = 8.8, 11.6$ Hz), 3.81–3.85 (m, 1H), 3.87 (s, 1H, OH), 4.24 (br t, 1H, $J = 10.6$ Hz), 5.05 (dt, 1H, $J = 1.5, 11.2$ Hz), 5.25 (dt, 1H, $J = 3.0, 11.2$ Hz), 5.43–5.46 (m, 1H), 5.68 (d, 1H, $J = 1.3$ Hz), 5.77 (s, 1H, NH); ^{13}C NMR (100 MHz, CDCl_3) δ 22.2, 24.0, 26.9, 28.7, 28.8, 30.9, 31.2, 31.4, 35.3, 35.8, 61.3, 62.5, 68.7, 97.8, 117.8, 127.4, 135.8, 154.5, 165.3, 174.7. All other latrunculin derivatives were prepared analogously.

Full details, including the structure and characterization of all intermediates, are provided in *Supporting Text*.

Results and Discussion

Strategic Considerations. The potential of natural product chemistry can be leveraged by diverting total synthesis projects to the preparation of "natural product-like" structures. This allows one not only to map pertinent SARs in great detail but may ultimately provide simplified yet fully functional analogues for applications in medicinal chemistry or chemical biology.

Our recent successful syntheses of **1** and **2** provide an optimal platform for such an endeavor (Scheme 1) (27, 28). More

important than being short and productive is their highly convergent character, which traces the targets back to three rather simple building blocks **A**–**C**. Because variations of these subunits are easily accomplished, the inherently modular character of our approach (29, 30)** lends itself to variations of all structural motifs of the natural products and may therefore pave the way for a better understanding of and control over the intriguing biological effects elicited by these compounds.

Toward this end, priority was granted to the following aspects. First, it seemed necessary to investigate the function of the conspicuous thiazolidinone ring which is a very rare heterocycle in nature but prominently featured in all naturally occurring latrunculins (2–12). Its role is particularly interesting in view of the x-ray structure of the Lat-A/actin complex (22), which unravels the hydrogen-bonding interactions between the protein and the macrolide ligand (Fig. 2). Specifically, the NH of **1** binds to aspartic acid (D157), the thiazolidinone carbonyl oxygen binds to threonine (T186), the C.17-OH binds to arginine (R210), the C.1 carbonyl oxygen binds to a water molecule that forms a bridge to glutamic acid (E214), and the C.13 ether oxygen binds to tyrosine (Y69). Interestingly, however, the crystal structure shows no immediate interactions between the S-atom below 3.5 Å except an intramolecular bond to the C.17 OH (3.478 Å). The two other closest residues are a water molecule (4.084 Å) and R206 (4.054 Å) (22). In view of this information, analogues were called for in which the S-atom is replaced by other heteroelements to gain a better understanding for the role of the thiazolidinone ring in actin binding. It is important to note that this fundamental aspect of the structure/activity profile cannot (easily) be probed by derivatization of the natural products.

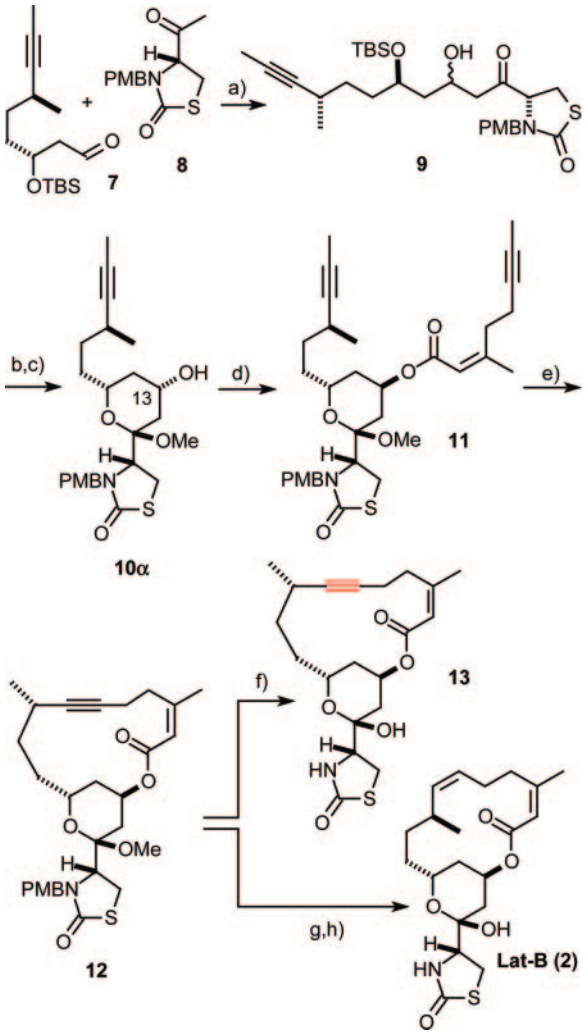
This is also true for the second structural feature of interest that concerns the function of the macrocycle. It is well established that latrunculin A (**1**) incorporating a 16-membered ring is more potent than its formally ring contracted congener **2** (6, 16). Although the C.5–C.7 segment of Lat-A shows little immediate contact to the protein environment but seem to reside in a hydrophobic pocket (22), it is reasonable to assume that changes in the length or functionalization of the aliphatic tether will modulate the cell permeability of the compound and affect the conformational rigidity of the bicyclic scaffold that exposes the attached thiazolidinone to the protein host. Consequently, nonnatural analogues were sought (*i*) that differ from the natural products in ring size and/or (*ii*) lack the methyl branches that are seemingly innocent but might be relevant for controlling the conformation of the rather stiff backbone, and (*iii*) in which one or both double bonds within the macrocycle are formally replaced by substructures that either increase or reduce the ring strain.

The third aspect of interest with regard to SAR concerns a stereochemical issue. A recent publication reports the surprising discovery that the 16-*epimer* of latrunculin B, i.e., compound **3**, is also a natural product (12). Because this stereochemical switch is supposed to affect the hydrogen bonding array, it seemed worthwhile to compare the activity of derivatives with both possible configurations at this chiral center.

Preparation of a Focused Library of Latrunculin Analogues. Our previous investigations have shown that ring-closing alkyne metathesis (31–34) combined with Lindlar hydrogenation is a highly adequate tool for the stereospecific formation of the macrocyclic rings in the parent compounds **1** and **2** (27, 28). This strategy, which is exemplarily outlined for **2** in Scheme 2, therefore constitutes a conserved design element en route to all analogues prepared herein.

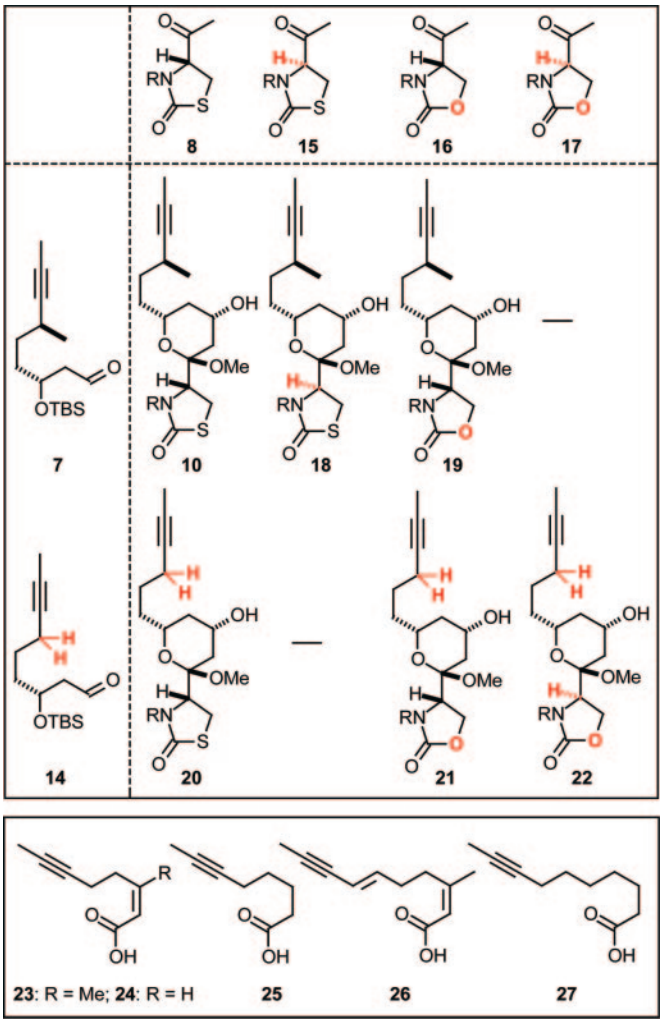
To obtain adequate analogues, two different aldehyde synthons (**7** and **14**) and four heterocyclic building blocks (**8** and **15**–**17**) were prepared and combined to give six out of the eight possible syntheses platforms of type **D** (**10** and **18**–**22**); the latter were then esterified with one of five acid segments (**23**–**27**) deemed appropriate for the purpose of this study (Scheme 3); their syntheses largely rely on methodology previously developed in this laboratory, most notably on iron-catalyzed cross coupling reactions (35–38). Exposure of the resulting diynes to catalytic amounts of either the tungsten alkylidyne complex $(t\text{BuO})_3\text{W}{\equiv}\text{CCMe}_3$ (**28**) (32, 39) or the molybdenum amide $[(t\text{Bu})(\text{Ar})\text{N}]_3\text{Mo}$ (**29**) activated *in situ* with CH_2Cl_2 as described in refs. 40–43 afforded the corresponding cycloalkynes (analogous to **12**; cf. Scheme 2) in decent to excellent yields; the latter were then elaborated by Lindlar hydrogenation and subsequent global deprotection with CAN following standard procedures (27).

In pursuing these routes, a set of 10 different latrunculin analogues was obtained in addition to the parent compounds latrunculin A (**1**) and B (**2**) serving as the calibration points for the envisaged biochemical assessment (Fig. 3). It is noteworthy that this synthesis campaign also completed the first total synthesis of 16-*epi*-latrunculin B (**3**), a natural compound on its own right as shown by a recent publication by Hoye *et al.* (12).



Scheme 2. Total synthesis of Lat-B (**2**) and its alkynyl analogue **13**. a, $TiCl_4$, $(i\text{Pr})_2\text{NEt}$, CH_2Cl_2, −78°C, 73%; b, aq. HCl, THF; c, camphorsulfonic acid, MeOH, 64% (over both steps); d, (*i*) triflic anhydride, pyridine, CH_2Cl_2, −10°C; (*ii*) Na salt of acid **23**, 15-crown-5, THF, 58%; e, $Mo[N(t\text{Bu})(3,5\text{-dimethylphenyl})]_3$ (**29**) (5 mol %), toluene, CH_2Cl_2, 80°C, 70%; f, CAN, $MeCN/H_2O$, 67%; g, H_2, Lindlar catalyst, CH_2Cl_2, quant.; h, CAN, $MeCN/H_2O$, 78%.

**For conceptually different total syntheses of Lat-A and Lat-B, see ref. 29 and 30.



Scheme 3. Matrix of six building blocks of type **D** prepared by aldol reactions of two different aldehydes with four different ketone segments (R = PMB), together with pertinent acid fragments used for the synthesis of the panel of latrunculin analogues depicted in Fig. 3.

Furthermore, the bare macrocycle **36** was prepared that constitutes a "negative control" for the importance of the five-membered heterocyclic ring.

As is evident from Fig. 3, this focused library comprises compounds with considerably different ring strain and greatly varying rigidities of the aliphatic segment spanning the conserved tetrahydropyran domain. Moreover, they show the desired modifications in the heterocyclic ring and belong to both epimeric series at C.16.

Evaluation of the Actin-Binding Properties. It is well established in the literature that incubation of nonmuscular cells with latrunculin derivatives triggers almost instantaneous morphological changes that constitute a convenient phenomenological read-out for actin binding (13, 16–20). This type of assay was used for the evaluation of the compounds summarized in Fig. 3, using the NIH/3T3 fibroblasts cell line, the actin cytoskeleton of which was stained with fluorescence-marked phalloidin (Fig. 4, micrograph I).

All compounds investigated, except the bare macrocycle **36**, were found to induce the depolymerization of F-actin at 10 μM concentration and become significantly cytotoxic at higher doses. This result shows that the implemented structural modifications are well accommodated and all nonnatural analogues remain functional.

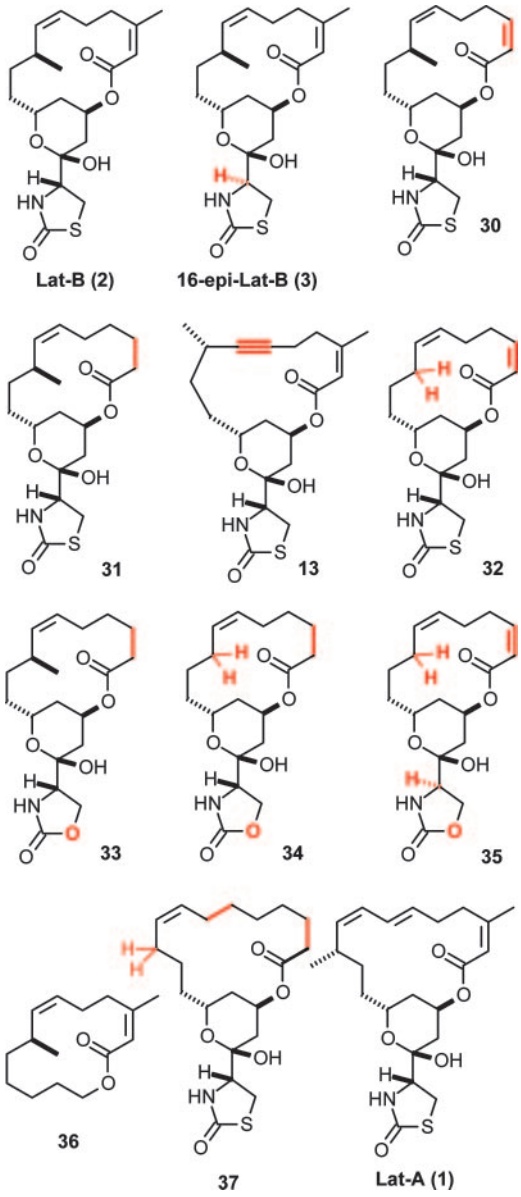


Fig. 3. Focused library of naturally occurring latrunculins and nonnatural analogues, all of which were prepared by total synthesis.

Among the three natural products included in the assay, the following order of activity was observed: Lat-A (**1**) > Lat-B (**2**) > 16-*epi*-Lat-B (**3**), in line with literature data (6, 16).

Having established the validity of the cellular assay with the natural products as calibration points, it was possible to assess the effect of the structural modifications on actin binding in more detail. In doing so, it was gratifying to find that the removal of the seemingly unfunctional methyl branches at C.3 and/or C.8 of the macrocyclic ring of **2** leads to a significant increase in potency. This effect is tentatively ascribed to the relaxation of the strained backbone, thus allowing for a better fit into the hydrophobic area of the binding pocket and exposure of the polar head groups in a more favorable way to the hydrogen-bonding partners of the protein host. Specifically, analogue **30** lacking the methyl branch at C.3 and even more so, the bis-*nor*-Lat-B derivative **32** clearly surpasses the natural lead compounds **2** and **3** in its effect on actin (**1** ≥ **32** > **30** > **2** > **3**; cf. Table 1). This is evident from the micrographs shown in Fig. 4, which compares the biological responses of the chosen cell line to the parent

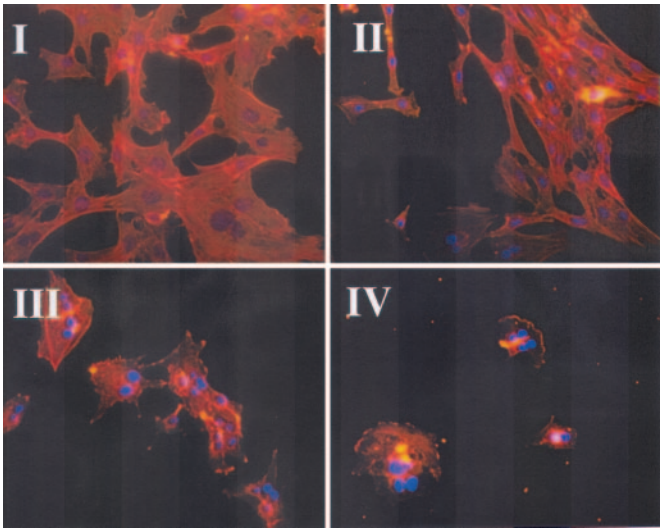


Fig. 4. Fluorescence micrographs (×250) of NIH 3T3 fibroblasts showing that compound **32** exerts a markedly stronger effect than the natural product Lat-B (**2**). The actin filament is stained with fluorescence-marked phalloidin, the nuclei with DAPI. (*I*) Untreated. (*II*) After incubation with **2** (5 μM). (*III*) After incubation with **32** (5 μM). (*IV*) After incubation with **32** (10 μM).

Table 1. Microfilament disrupting activity of Lat-A (1), Lat-B (2), 16-*epi*-Lat-B (3), and fully synthetic analogues

	Effective concentration, μM		
Compound	1	5	10
1	+	++	+++
2	−	+	++
3	−	±	+
30	±	+	++
32	+	++	+++
31	−	±	+
13	−	−	+
33	−	±	+
34	−	±	+
35	−	+	±+
37	−	±	+
36	−	−	−

−, no effect; ±, weak effect (unaltered cell number, intact cell morphology, filamentous and slightly disrupted actin); +, significant effect (<80% viable cells, spindle-shaped cell morphology, filamentous and disrupted actin); ++, strong effect (<50% viable cells, poly-nuclear cells, more disrupted than filamentous actin); +++, very strong effect (<20% viable cells, poly-nuclear, rounding and isolated cells, disrupted actin).

compound **2** and its nonnatural analogue **32** at two different concentrations. These pictures clearly show that compound **32** reaches a level of activity previously known for latrunculin A (**1**) only, which is the most potent member of this series (6, 16). Because **32** is much easier to prepare than both **1** and **2** by a route that has only 11 steps in the longest linear sequence (for details, see *Supporting Text*), this particular analogue may become available through total synthesis in sufficient quantity at a competitive price and might therefore help to ensure a constant supply of a fully functional and potent actin-binding probe molecule.††

The other structural modifications engender a *slight* decrease in activity. Specifically, all compounds belonging to the 16-*epi*-series are somewhat less active than their "naturally configured" analogues. The same is true for the compounds in which the rather unique thiazolidinone ring is replaced by an oxazolidinone entity. Although the S-atom does not seem to entertain a direct hydrogen bond to any of the heteroelements of the protein (vide supra), it seems to be better accommodated by the host. Whether this is due to its larger size and better polarizibility remains to be elucidated. Note, however, that the potency of all of these synthetic analogues is in the same range as that of the naturally occurring 16-*epi*-Lat-B (**3**) (cf. Table 1).

Conclusions. Whereas essentially all post facto chemical derivatizations of latrunculin A or B reported in the literature led to a *complete loss* of the actin-binding capacity of these marine natural products (24–26), a series of fully functional synthetic analogues is now presented, some members of which even surpass the natural lead compounds in terms of bioactivity. Because none of the synthetic analogues is available from the natural products without unreasonable effort, this study highlights the enabling power of the concept of "total synthesis-driven" searches for SAR profiles of complex bioactive molecules. One of the new compounds, i.e., product **32**, might even qualify as a substitute for **1** and **2** in future studies, because its synthesis is short, convergent, and productive and its activity rivals that of the most active member of the latrunculin family (**1** ≈ **32**). *De novo* synthesis of **32** might therefore be an alternative to the inherently problematic isolation of the latrunculins by extracting sponges harvested in highly sensitive coral reef environments.

The finding that certain simplifications of the gross structure of the latrunculins go hand-in-hand with an increase in potency constitutes a particularly noteworthy and synthetically relevant aspect. It is likely that this effect stems from the relief of strain of the 14-membered backbone of Lat-B. Moreover, it has been shown that the conspicuous thiazolidinone ring present in all naturally occurring latrunculins may be optimal for actin binding but is definitely not essential because it can be replaced by an oxazolidinone moiety with only slight losses in efficacy. Likewise, the absolute configuration of the chiral center C.16 plays a minor role with regard to the bioactivity of these exquisite probe molecules. These insights provide valuable guidance for further investigations in this field.

We thank Dr. L. Parra-Rapado for preparing the bare macrocycle **36**, Mrs. Anette Langerak for excellent technical assistance, and Prof. H. Waldmann for generous support of our collaborative work. This work was supported by the Max-Planck-Gesellschaft (Chemical Genomics Center Initiative), the Deutsche Forschungsgemeinschaft (Leibniz award to A.F.), an Alexander-von-Humboldt Foundation fellowship (to M.D.B.F.), the Merck Research Council, and the Arthur C. Cope Scholar Funds administered by the American Chemical Society.

††Access to the latrunculins by harvesting and extracting the producing sponge becomes increasingly difficult. Therefore, first attempts to grow *N. magnifica* in aquacultures have been reported (cf. ref. 5).

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