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REVIEW ARTICLE

Chemotherapeutics overcoming nonsense mutation-associated genetic diseases: medicinal chemistry of negamycin

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Nonsense mutations caused by the presence of an in-frame premature termination codon (PTC) account for ~ 10% of gene lesions that together cause over 1800 inherited human diseases. One approach to treating genetic diseases that stem from PTCs is selective promotion of translational readthrough in a PTC using 'readthrough compounds' that can lead to partial restoration of full-length functional protein expression. (+)-Negamycin, a natural dipeptide-like antibiotic, may restore some dystrophin expression in the skeletal muscles of mice with Duchenne muscular dystrophy, and this compound has been recognized as a potential therapeutic agent for diseases caused by nonsense mutations. In an effort to develop new candidate molecules with improved activities, we established the efficient total synthesis in eight steps of (+)-negamycin using both achiral and chiral starting material. These routes provided a deamino derivative with *in vivo* readthrough activity with potential for long-term treatment. In a separate approach, we discovered two natural negamycin analogs, 3-*epi*-deoxynegamycin and its leucine derivative, which are potent readthrough compounds effective against nonsense mutations of eukaryotes but not prokaryotes. These compounds fail to display antimicrobial activity. More potent derivatives, whose structure is derived from 3-*epi*-deoxynegamycin, were identified and their chemistry is discussed in this review.
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INTRODUCTION

Hereditary diseases are often caused by genetic abnormalities. Following recent developments in genome science and the use of next-generation DNA sequencers in personalized medicine, it was established that some 5–20% of genetic diseases could be attributed to nonsense mutations.[1] Nonsense mutation-associated diseases, also known as nonsense diseases, are caused by point mutations that form premature termination codons (PTCs) in a structural gene. PTCs interfere with the expression of a functional protein. To date, more than 1800 nonsense diseases have been identified.[2]

As a representative hereditary disease, Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disease that occurs in 1/3500 newborn boys.[3] About 10–20% of DMD cases are caused by a nonsense mutation in the dystrophin gene, which encodes dystrophin, the dystrophin protein. In the absence of dystrophin, the crosslinking structures of the muscle cell membrane and the extracellular basement membrane, which form the muscle cell skeleton, are broken, and myocyte disorder and necrosis progress.[4] Steroid administration is currently used to treat symptoms and prolong life, but a cure for the disease is yet to be found. In another disease, cystic fibrosis, a nonsense mutation present in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) results in the abnormal secretion of chloride ions into the gastrointestinal tract and respiratory tract. This causes intestinal malabsorption and obstruction, and respiratory tract infections.[5] European and American Caucasians suffer from cystic fibrosis at a rate of 1 in 2500.[6] Many types of cancers are caused by genetic disorders. In cancer patients, recent studies have found mutations in the tumor suppressor gene *p53*, which is involved in apoptosis induction, in more than 50% of the subjects. Floquet *et al.*[7] have reported that 8% of these mutations are nonsense mutations.

One therapeutic approach to regeneration of proteins lost due to the presence of nonsense mutations involves gene transfer techniques based on viral vectors or stem cell therapies.[8,9] These methods face several hurdles, including the production of safe vector systems, and ethical and social considerations have also hindered the development of gene transfer therapies. No fundamental therapies have yet been developed for the treatment of diseases caused by nonsense mutations. In particular, dystrophin gene is the largest among known human genes, and any vectors remain largely unexplored at the delivery of full-length dystrophin gene. However, it is reported that adeno-associated virus vector containing mini/micro dystrophin gene can partially produce the functional dystrophin protein.[10] Moreover, recently, the CRISPR/Cas9 system directly edited the genome of dystrophin in muscle and muscle stem cells in mice, resulting in the production of dystrophin protein.[11] On the other hand, a few chemotherapeutic approaches have recently been studied. Among

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these are molecules that can enable the ribosomal machinery to skip a PTC during translation, resulting in the expression of a full-length functional protein (Figure 1).[12] Such molecules are called 'readthrough drugs' and represent an attractive strategy for overcoming nonsense mutation diseases. The mechanism underlying PTC readthrough has not been elucidated to date, and additional research studies and medicinal chemistry efforts are needed. The development of specific drugs for each of the ~1800 nonsense mutation-based genetic diseases will require a tremendous effort and one way for drug development is the creation of drugs that are applicable to a variety of genetic diseases. The development of readthrough drugs could make a meaningful contribution to treatment of diseases with a high unmet medical need.

READTHROUGH COMPOUNDS

Most readthrough compounds are thought to bind directly to ribosomal RNA[12] and some examples of such compounds are shown in Figure 2. Aminoglycoside antibiotics, such as gentamicin (**1**), are typical readthrough compounds. In 1999, Barton-Davis *et al.*[13] demonstrated that **1** provides readthrough over a PTC in the mRNA of dystrophin in a mouse model of DMD such as *mdx* mice restoring dystrophin expression to 10% of the normal level. In 2002, Du *et al.*[14] demonstrated that **1** can restore the synthesis of functional hCFTR protein by suppressing the hCFTR-premature stop mutation in transgenic mice. Although **1** is a potent readthrough compound, it cannot be administered over long periods of time because of serious side effects common to many aminoglycosides. These side effects include nephrotoxicity[15] and ototoxicity.[16] Long-term aminoglycoside treatment is also not advisable because of the potential development of drug-resistant bacterial infections. Some molecules, such as G418 (**2**), the most potent readthrough aminoglycoside, display strong cellular toxicity.[17] Consequently, aminoglycosides are considered to be poor readthrough drug candidates. Medicinal chemistry approaches applied to aminoglycosides have identified potent readthrough compounds with more acceptable side effect profiles. For example, Nudelman *et al.*[18] reported the characterization of an aminoglycoside analog, NB 54 (**3**), which displays more potent readthrough behavior than gentamicin. In an *in vivo* acute toxicity test, the LD_{50} of **3** was found to be significantly lower than that of **1**. The same group recently discovered NB124 (**4**), a synthetic aminoglycoside derivative that strongly induces apoptosis in human tumor cells by promoting high levels of PTC readthrough, thus contributing to the production of a full-length p53 protein capable of activating *p53*-dependent genes.[19] Recently, arbekacin (**5**), an aminoglycoside that contains an (*S*)-4-amino-2-hydroxybutanoyl group at the central cyclohexane ring and has relatively low side effects,[20] was found to provide satisfactory readthrough activity.[21] Investigators at four medical institutions in Japan have initiated clinical trials of arbekacin.[22]

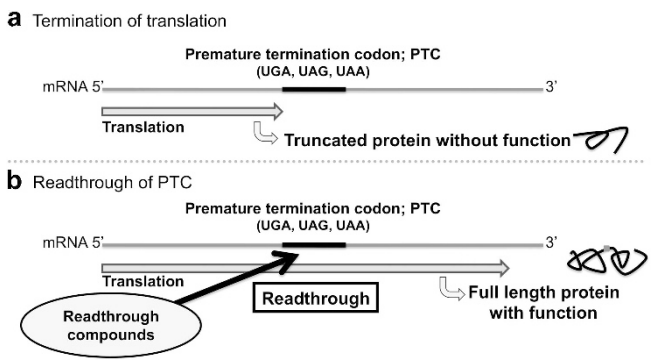


Figure 1 The readthrough of premature termination codons on mRNA in the presence of readthrough compounds. (**a**) Termination of translation by the existence of PTC; (**b**) Readthrough of PTC by readthrough compounds.

Non-aminoglycoside readthrough compounds have also been identified. For example, ataluren (PTC124, **6**), a 1,2,4-oxadiazole derivative discovered by high-throughput screening of a chemical library, has been found to promote the accumulation of dystrophin in the tibias anterior muscle, the diaphragm and the myocardium of orally treated *mdx* mice.[23] Clinical trials of this compound have been completed, and both Phase 2a and 2b studies in DMD showed that **6** increases dystrophin levels in the muscle biopsies of patients and, compared with a placebo, slightly reduces disease progression.[24] PTC124 (**6**) has been conditionally approved by the European Medicines Agency as a new DMD chemotherapy. Further validation of its therapeutic index and safety profile are awaited for a Phase 3 trial. A Phase 3 clinical trial involving cystic fibrosis chemotherapy revealed that **6** fails to restore CFTR function.[25]

Through an assay for identification of novel PTC readthrough compounds using ataxia-telangiectasia (spider veins) as a genetic disease model, RTC#13 (**7**) and RTC#14 (**8**) have been developed.[26] It has also been reported that Amlexanox (**9**), which has anti-allergic[27] and anti-inflammatory[28] properties, not only induces an increase in the amount of nonsense containing mRNAs in treated cells, but also leads to the efficient synthesis of the full-length protein.[29]

In an effort to develop a readthrough chemotherapy candidate, we focused on a low-MW natural antibiotic (+)-negamycin [(2-[(3*R*, 5*R*)-3,6-diamino-5-hydroxyhexanoyl]-1-methylhydrazino)acetic acid] (**10**, Figure 2) isolated by Umezawa *et al.* in 1970 from the culture filtrates of three strains related to *Streptomyces purperfuscus*.[30] **10** exhibits a low acute toxicity and strong inhibitory activity against multiple drug-resistant enteric Gram-negative bacteria, including *Pseudomonas aeruginosa*.[31] The antimicrobial activity of **10** is derived from a genetic miscoding in the bacterial ribosomal system, which leads to the specific inhibition of protein biosynthesis. Because miscoding causes the readthrough of termination signals, considerable attention has been focused on **10** as a potential therapeutic agent against nonsense diseases. For example, Arakawa *et al.* reported that negamycin restores dystrophin expression in the skeletal and cardiac muscles of *mdx* mice.[32] We adopted **10** as a lead compound for readthrough drug development and have initiated a drug discovery study. In this review, we highlight our recent achievements in the synthesis and medicinal chemistry of negamycin, including the results of extensive structure–activity relationship (SAR) studies and the discovery of natural negamycins as potential readthrough drugs.

TOTAL SYNTHESIS OF NEGAMYCIN (10)

Several total syntheses of **10** have been reported since 1972.[33–46] Negamycin displays unique biological activity and is a fascinating synthetic target molecule for chemists because of the existence of a unique and unusual amino-acid residue and four functional groups and two chiral centers on the 5-carbon side chain. The first total synthesis of **10** was reported in 1972 from D-galacturonic acid and the assigned structure of the natural product was confirmed.[33] Both racemic and optically active versions of **10** were obtained in moderate overall yields in other syntheses. An efficient shorter synthetic route to **10** and its derivatives may be significant for the development of promising new therapeutic candidates for DMD and other diseases caused by nonsense mutations.

We established a rapid and efficient synthetic route including two asymmetric reactions for the total synthesis of **10** from N-Boc-glycinal (**16**), an achiral starting material.[47] As shown in Scheme 1, based on

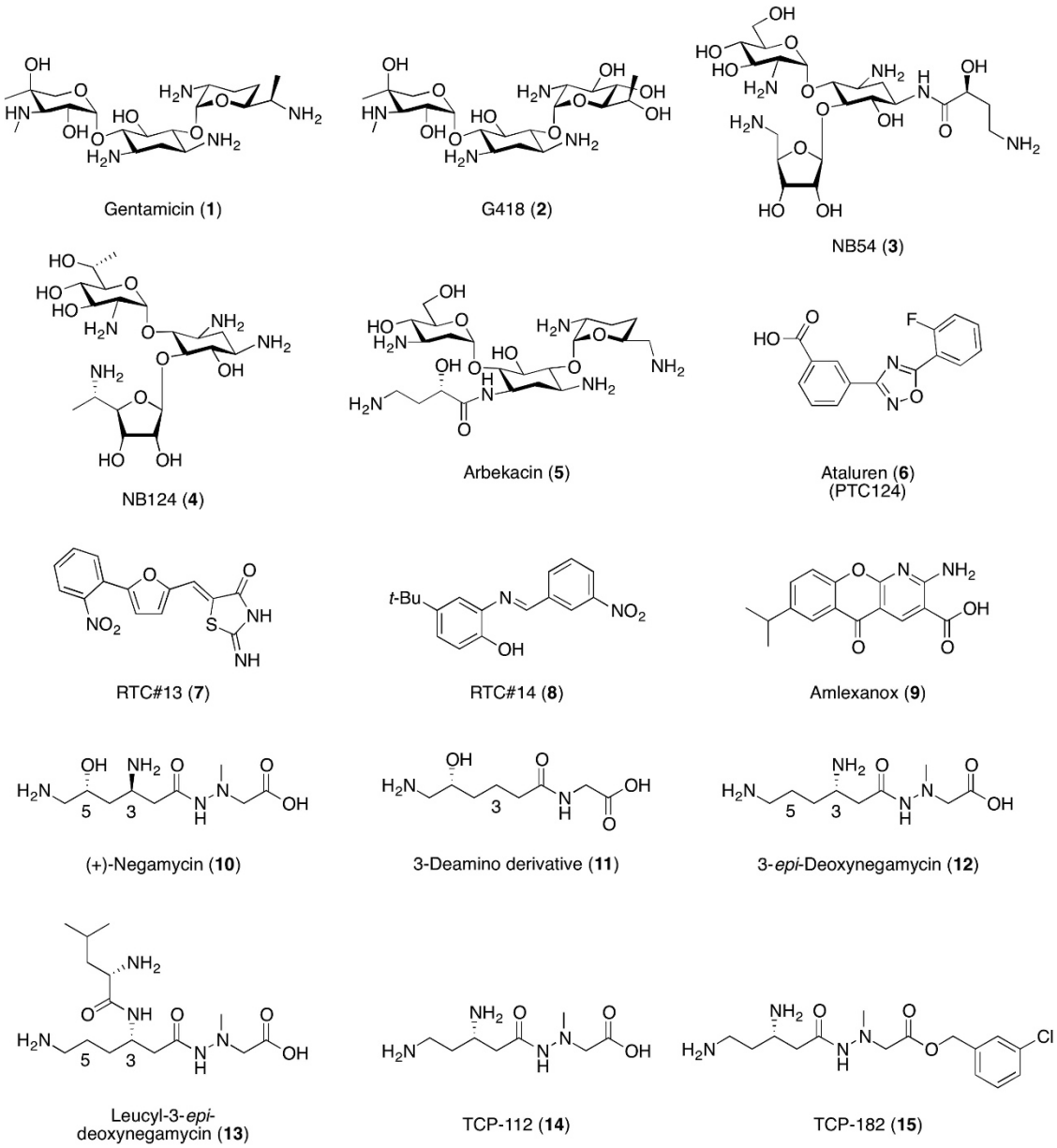
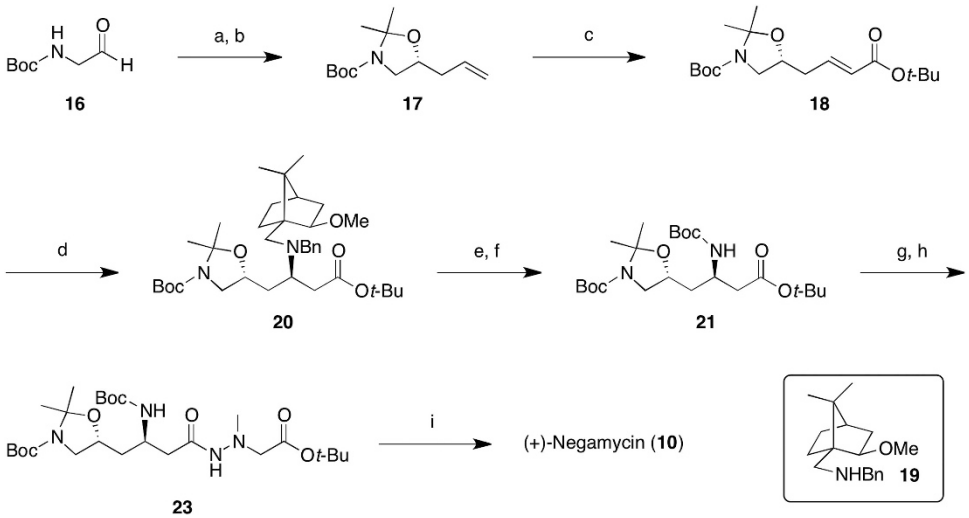
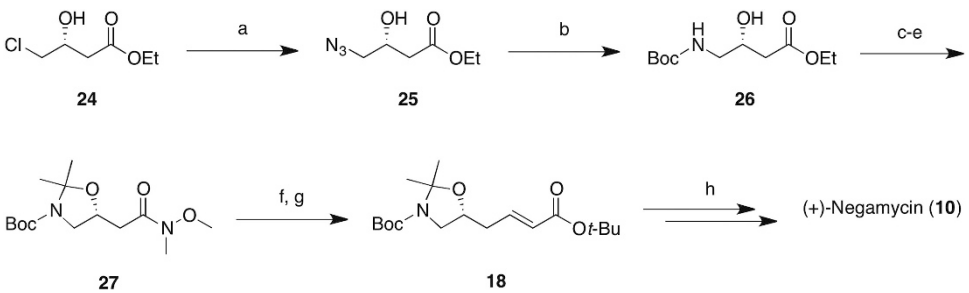


Figure 2 Structures of low-MW compounds with readthrough activities.

Brown's procedure,[48] an asymmetric allylboration using (+)-B-allyl-diisopinocampheylboran [(+)–Ipc_2B (allyl)] was applied to **16**, and the resulting amino alcohol was immediately protected as a 2,2-dimethyl-oxazolidine by treatment with 2,2-dimethoxypropane and boron trifluoride-diethyl ether complex ($BF_3{\cdot}Et_2O$) to obtain **17** in high yield (90%). The oxazolidine (**17**) was condensed with *tert*-butyl acrylate by using Grubbs' second generation as a catalyst and microwave irradiation to afford the α,β-unsaturated *tert*-butyl ester **18** with the (E) olefinic configuration in 83% yield.[49,50] The desired key intermediate (**18**) was thus easily prepared in two steps from the achiral starting material **16**. Next, a chiral amine corresponding to that at the C3 position on the left side of **10** was introduced into the intermediate **18** by an asymmetric Michael addition using methoxybornyl-10-benzyl-amine (**19**)[51] in the presence of a base to give the Michael adduct (**20**) as the sole diastereomer (de > 99%) in high yield (80%). After the *N*-benzyl group and the chiral auxiliary of **20** were oxidatively removed using *N*-iodosuccinimide in CH_2Cl_2, the resulting free amino group was immediately protected by a Boc group in the presence of Et_3N. Then, the *tert*-butyl ester of the protected β-amino acid (**21**) was hydrolyzed under basic conditions (2 M KOH/MeOH), and this was followed by coupling with a hydrazine·*p*-toluenesulfonic acid salt **22** using a 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-1-hydroxybenzotriazole (EDC–HOBt) method to afford the hydrazide (**23**). Finally, all the protecting groups in **23** were removed by treatment with 4 M HCl/dioxane. The crude product obtained in this way was purified by ion exchange chromatography on Amberlite CG50 (NH_4^+ form) to afford the target (**10**) in 98% yield. The chemical data, including ^1H, ^{13}C NMR, HRMS and optical rotation, obtained from the synthetic **10** were identical to the data obtained from the natural product. These results indicated that the total synthesis of the optically active **10** was achieved with a total yield of 42% over eight steps. This synthetic route provides the shortest,



Scheme 1 Efficient total synthesis of (+)-negamycin (**10**). Reagents and conditions: (a) (+)-Ipc$_2$B(allyl), Et$_2$O, −100 °C, then 1 N NaOH aq. H$_2$O$_2$, reflux; (b) 2,2-dimethoxypropane, BF$_3$·Et$_2$O, acetone, reflux, 90% (two steps); (c) *t*-butyl acrylate, Grubbs' catalyst second generation, CH$_2$Cl$_2$, microwave, 40 °C, 83%; (d) **19**, *n*-BuLi, tetrahydrofuran (THF), −40 °C, 80%; (e) *N*-iodosuccinimide, CH$_2$Cl$_2$, room temperature (rt); (f) (Boc)$_2$O, Et$_3$N, THF, 0 °C to rt, 80% (two steps); (g) 2 M KOH/MeOH, microwave, 100 °C; (h) PTSA·H$_2$NN(Me)CH$_2$CO$_2$*t*-Bu (**22**), Et$_3$N, HOBt·H$_2$O, EDC·HCl, CH$_2$Cl$_2$, 0 °C to rt, 98% (two steps); (**i**) 4 M HCl/dioxane, 0 °C, then ion exchange chromatography, 98%.



Scheme 2 Formal total synthesis of (+)-negamycin (**10**). Reagents and conditions: (a) NaN$_3$, DMF, 100 °C, 90%; (b) H$_2$, Pd/C, (Boc)$_2$O, AcOEt, 67%; (c) KOH, EtOH/H$_2$O = 2:1, rt, 99%; (d) HCl·HN(OMe)Me, Et$_3$N, EDC·HCl, DMF, 0 °C to rt, 92%; (e) 2,2-dimethoxypropane, BF$_3$·Et$_2$O, acetone, reflux, 95%; (f) DIBAL-H, toluene, −78 °C; (g) Ph$_3$P = CHCO$_2$*t*-Bu, THF, reflux, 80% (2 steps). (h) Same six steps from the intermediate (**18**) to negamycin (**10**) as are shown as Scheme 1.

most efficient synthesis to negamycin identified since the first total synthetic route was reported 40 years ago.

Synthetic diversity leading to negamycin analogs was secured by developing a formal synthesis of **10** through an improved synthesis of the key synthetic intermediate (**18**) from the commercially available chiral compound (**24**; Scheme 2).[52] The synthetic route begins with the substitution of a chloride for an azide group in dimethylformamide (DMF), a reaction that proceeds with 90% yield. The Boc-protected compound **26** was efficiently obtained in 67% yield in a one-pot reaction involving the reduction of the azide (**25**) under hydrogen in the presence of Pd/C (10%) and protection of the amine by Boc$_2$O. After the ethyl ester of **26** was hydrolyzed and condensed with *N,O*-dimethylhydroxylamine·HCl, the hydroxyl group and amide nitrogen of the resulting Weinreb amide were protected with 2,2-dimethoxypropane to give the oxazolidine (**27**) in 87% yield over three steps. Then, **27** was reduced to the aldehyde by DIBAL-H, and this was followed by a Wittig reaction with (*tert*-Boc-methylene)triphenylphosphorane to give the key intermediate (**18**) in 80% yield in two steps. This synthesis provides the key intermediate (**18**) in 42% yield over seven steps from the chiral starting material (**24**). In view of the cost of Grubbs catalyst, this proved to be a preferable route to the key intermediate (**18**) in quantities useful for the subsequent synthesis of negamycin analogs. Therefore, we adopted the alternative formal synthetic route of (+)−**10**, starting from the commercially available chiral ester (**24**), involving 13 steps with an overall yield of 31%.

DETERMINATION OF READTHROUGH ACTIVITY

The readthrough activities of the various analogs were evaluated using an *in vitro* (in cell or cell-free) or *in vivo* dual-reporter gene expression system involving a PTC (TGA, TAG or TAA) between the β-galactosidase and luciferase gene sequences. The *in vitro* system was constructed based on an *in vivo* system developed by Shiozuka *et al.*[53] As shown in Figure 3, β-galactosidase is positioned upstream of the PTC and is expressed constitutively in the reporter assay system. Luciferase, positioned downstream of the PTC, is expressed when PTC readthrough occurs. Measuring both enzymatic activities in the cell lysate or tissue homogenates provides the readthrough activities of the analogs as the ratio between the luciferase activity and the β-galactosidase activity.

SAR STUDY OF 10: THE C3 POSITION

Our efforts then shifted from the medicinal chemistry of negamycin (**10**) toward the development of analogs with potent readthrough

activity. In order to understand the importance of the amino group at the C3 position of **10**, a series of analogs **11**, **28–33** were first designed and synthesized using the aforementioned two synthetic routes. The *in vitro* evaluation system had not yet been established, and so the biological activity of these analogs was measured by an *in vivo* readthrough assay using transgenic mice (READ mice)[53] bearing the dual-reporter gene described in Figure 3. As shown in Figure 4b,

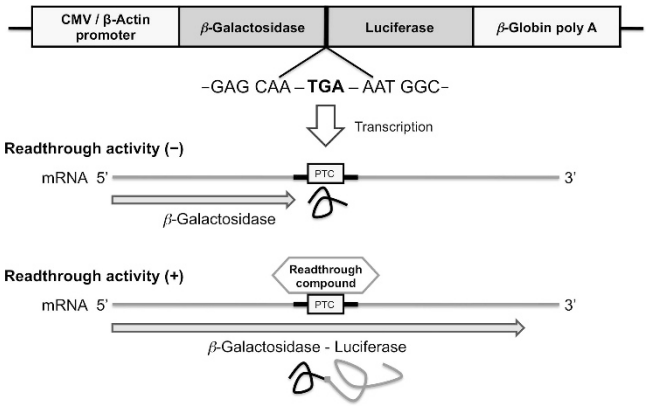
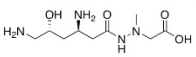
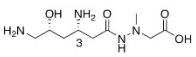
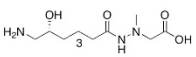
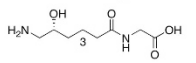
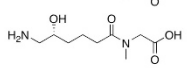
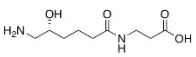
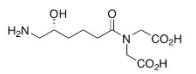
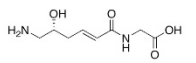


Figure 3 The dual-reporter system used to evaluate the readthrough activity.

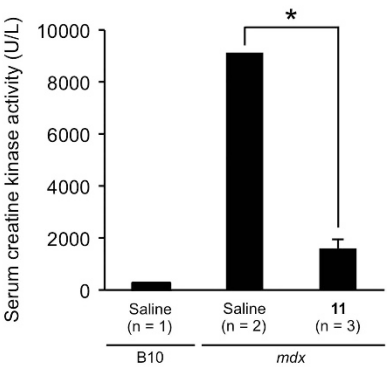
(+)-3-*epi*-negamycin (**28**), a C3 epimer of **10**, was found to exhibit a readthrough activity comparable to **10**, whereas **29**, which lacks the amino group, displays lower readthrough and antimicrobial activities. These results indicate that the presence of the three-amino group is important for both activities. Interestingly, **11**, from which both the *N*-methyl and amino groups have been removed, exhibits a 1.4-fold higher readthrough activity than **10** but shows no antimicrobial activity. However, it possesses distinct readthrough and antimicrobial activities. The analogs **30–33**, based on the chemical structure of **11**, show no significant readthrough activity.

The biological effects of **11** were further explored by evaluation of its biochemical and immunohistochemical properties. For biochemical evaluation, we assessed the levels of serum creatine kinase,[54] a clinical marker of DMD, in *mdx* mice administered a daily s.c. injection (1 mg per day per 20 g bodyweight for 4 weeks). The serum creatine kinase level in **11**-treated *mdx* mice is significantly suppressed compared with that of the untreated mice (Figure 4b). This result suggests that **11** enhances the strength of the muscle fibers by increasing the expression of functional dystrophin. An immunohistochemical evaluation of **11** was performed by staining dystrophin in muscle tissues after daily injection of **11** over 4 weeks. Dystrophin expression in the skeletal muscle of B10 mice can be clearly observed (Figure 4c–i), whereas untreated *mdx* mice lack this signal (Figure 4c–ii). In contrast to these controls, dystrophin expression in the skeletal muscle of **11**-treated

a

Compound	Structure	Readthrough activity[a]	Antimicrobial activity[b]
(+)-Negamycin (**10**)		1.00 ± 0.25	32/128/2/32/8
28		1.01 ± 0.16	>50/>50/25/>50/50
29		0.83 ± 0.13	>128
11		1.36 ± 0.14	>128
30		0.92 ± 0.09	Not tested
31		< 0.8	>128
32		< 0.8	Not tested
33		< 0.8	>128

b



c

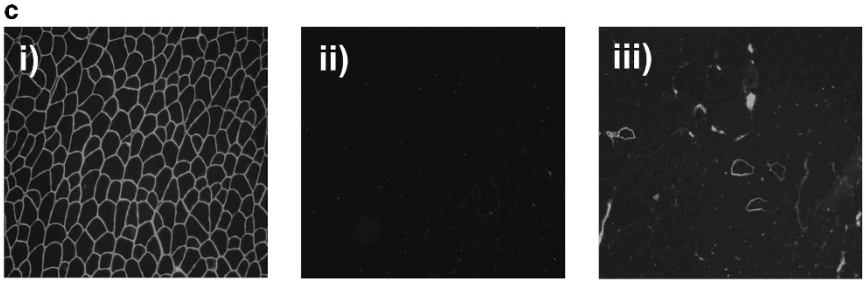


Figure 4 (**a**) The biological activities of analogs, with focus on the amino group. [a]Relative *in vivo* readthrough activity compared with that of gentamicin (=1). Analogs were subcutaneously injected into the abdominal region of a READ mouse at a dose of 0.1 mg per day per 20 g bodyweight for 7 days. Data represent the mean ± s.d. (n=4). [b]The antimicrobial activities (MIC, μg ml^{-1}) against several microorganisms (*Staphylococcus aureus* FDA 209 P/*Bacillus subtilis* NRRL B-558/*Escherichia coli* BEM11/*Shigella dysenteriae* J S11910/*Pseudomonas aeruginosa* A3). (**b**) Serum creatine kinase (CK) levels in *mdx* mice. Statistical analysis was performed using a Student's *t*-test. *P=0.001. Error bars indicate ± s.d. (**c**) Dystrophin expression in skeletal muscles, (i) normal B10 mouse, (ii) untreated *mdx* mouse, (iii) **11**-treated *mdx* mouse. A full color version of this figure is available at The *Journal of Antibiotics journal* online.

Table 1 The biological activities of the negamycins and aminoglycoside G418 (2)

	Readthrough activity[a]			
Compound	*–TGA–*	*–TAG–*	*–TAA–*	*Antimicrobial activity*[b]
(+)-Negamycin (**10**)	1.51±0.06	1.45±0.19	1.35±0.08	⩾1024/512/64/128
3-*epi*-DN (**12**)	2.51±0.09	1.20±0.38	0.62±0.11	⩾1024
Leucyl-3-*epi*-DN (**13**)	2.56±0.02	1.81±0.04	1.50±0.03	⩾1024
G418 (**2**)	5.85±0.12	8.18±0.41	3.77±0.08	Not tested[c]

Abbreviation: DN, deoxynegamycin.
[a]Cell-based readthrough activity compared with control (=1) in COS-7 cells. The compounds were evaluated at a concentration of 200 μM. Data represent the mean±s.d. (n=3).
[b]Antimicrobial activity (MIC, μg ml^{-1}) against several microorganisms (*Staphylococcus aureus* ATCC29213/*Bacillus subtilis* ATCC6633/*Escherichia coli* ATCC25922/*Pseudomonas aeruginosa* ATCC27853).
[c]Antimicrobial activity (MIC, μM) of G418 against *E. coli* DH5α and *B. subtilis* ATCC6633 were 6.3 and <1.25, respectively.[57,58]

mdx mice is partially restored (Figure 4c–iii). The three-deamino derivative (**11**) appears to promote PTC readthrough in the dystrophin gene, resulting in the production of a dystrophin protein. Moreover, **11** exhibits a lower *in vivo* toxicity profile than **10**.[55] **11** is, therefore, a potential therapeutic candidate for DMD.

SAR STUDY OF 10: THE C3 AND C5 POSITIONS

Using an alternative approach for the development of new readthrough compounds, we became interested in two natural products related to negamycin: 3-*epi*-deoxynegamycin (**12**) and leucyl-3-*epi*-deoxynegamycin (**13**; Figure 2). These 5-deoxy analogs were originally isolated in 1977 from the culture broth of *Streptomyces* No. MD967-A2, a strain of *S. goshikiensis*.[56] Their biological activities were unknown for several years, but they were known to have almost no antimicrobial activity compared with (+)-negamycin (**10**).

The possibility that these products might be useful as readthrough compounds was investigated by synthesizing them and evaluating their *in vitro* readthrough activities using a cell-based or a cell-free protein synthesis system. First, the cell-based readthrough activity of the negamycins **10**, **12**, **13** and G418 (**2**) against three types of PTCs and the antimicrobial activity of them[57,58] were measured (Table 1). As a positive control, **10** was found to exhibit comparable readthrough activities (1.3–1.5) for each PTC. Surprisingly, the readthrough assay of a TGA-type PTC revealed that the synthesized natural analogs (**12**, **13**) show higher activities (2.51 and 2.56, respectively) than **10**. Moreover, in the case of the TAG and TAA PTCs, **12** shows a readthrough activity comparable to that of **10** against TAG, but it shows no activity against TAA. Compound **13**, a leucine adduct of **12** at the 3-position through an amide bond, displays low readthrough activity against both TAG and TAA, but these activities are slightly higher than the activities of **10**. It is possible that the leucine residue on **13** slightly improves the readthrough activities against TAG and TAA.

The negamycins all tend to display TGA readthrough activities, whereas the aminoglycoside G418 (**2**) displays TAG activity, suggesting that the readthrough mechanisms underlying these compounds are different. This hypothesis is supported by the fact that **12** and **13** do not show any antimicrobial activity against several Gram-positive or -negative bacteria (MIC⩾1024 μg ml^{-1}), as reported previously by Kondo *et al.*[56] Slight structural changes in **10** appear to disrupt the antimicrobial activity and separate the readthrough and antimicrobial activities.[55,59] Recently, an X-ray crystallographic analysis of **10** complexed with prokaryotic ribosomes revealed that **10** binds to the small subunit head domain of the *Escherichia coli* ribosome and both small and large ribosomal subunits of the *Thermus thermophiles*.[60,61] Our discovery suggested that eukaryotic ribosomes may recognize **10** and its other analogs with readthrough activity. By contrast, prokaryotic ribosomes strictly recognize the chemical structure of **10**, and, among the negamycins, only **10** shows significant antibacterial activity.

Table 2 Readthrough activities of negamycins in cell-free protein synthesis

	Readthrough activity[a]		
Compound	*–TGA–*	*–TAG–*	*–TAA–*
Eukaryotic protein synthesis system			
(+)-Negamycin (**10**)	2.05±0.02	1.34±0.14	1.24±0.02
3-*epi*-DN (**12**)	3.40±0.16	Not tested	Not tested
Leucyl-3-*epi*-DN (**13**)	4.54±0.02	1.48±0.05	1.60±0.03
Prokaryotic protein synthesis system			
3-*epi*-DN (**12**)	0.63±0.02	0.93±0.11	0.45±0.03
Leucyl-3-*epi*-DN (**13**)	0.77±0.07	0.81±0.03	0.38±0.02

Abbreviation: DN, deoxynegamycin.
The compounds were evaluated at a concentration of 20 μM. Data represent the mean±s.d. (n=3).
[a]Readthrough activity as compared with control (=1).

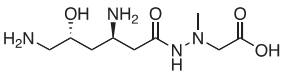
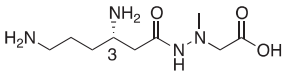
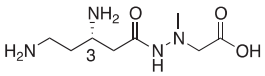
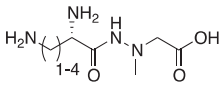
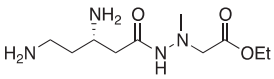
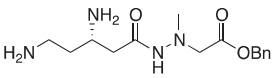
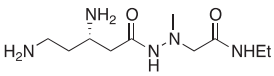
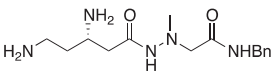
The effects of the negamycins on the eukaryotic and prokaryotic translation of a nonsense-mutated gene were tested using a cell-free protein synthesis system that can directly evaluate the readthrough activity. The assay was staged using a lysate derived from a human cell line and *E. coli* extract. As shown in Table 2, the eukaryotic systems display similar activities to those obtained from the cell-based readthrough assays. These values corroborate our cell-based assay results. The direct readthrough activity of **13** against TGA is strong, with a value of 4.54.

On the other hand, no readthrough activity was observed for any of the compounds in prokaryotic systems. These results suggest that the natural products are unlikely to contribute to prokaryotic survival strategies in the context of nonsense mutations. These compounds may therefore contribute to the development of selective readthrough agents in eukaryotes that may be useful as chemotherapies for the treatment of human genetic diseases caused by nonsense mutations. We succeeded in discovering valuable lead compounds for the development of selective readthrough drugs, which do not promote the emergence of resistant bacteria.[62]

SAR STUDY OF 3-*EPI*-DEOXYNEGAMYCIN (12)

3-*Epi*-deoxynegamycin (**12**, Table 3) was selected as a simpler lead compound with potent readthrough activity, a replacement for

Table 3 Cell-based readthrough activities of (+)-negamycin (10) and synthetic derivatives

Compound	Structure	Readthrough activity[a]
(+)-Negamycin (**10**)		1.81 ± 0.17
3-*epi*-DN (**12**)		2.94 ± 0.23
TCP-112 (**14**)		4.28 ± 0.16
34a-d		0.99–1.30
35		2.08 ± 0.11
36		4.04 ± 0.06
37		1.42 ± 0.15
38		0.80 ± 0.05

Abbreviation: DN, deoxynegamycin.
Compounds were evaluated at a concentration of 200 μM. Data represent the mean ± s.d. ($n=3$).
[a]Readthrough activity relative to control (=1) in COS-7 cells.

negamycin. We next developed a SAR study to identify novel potent derivatives while focusing on the following features: (i) the length of the carbon chain in the β-amino-acid residue; (ii) the position of the 3-amino group on the carbon chain (2 or 3); and (iii) modification of the carboxylic acid.[63]

The readthrough activities of the synthetic analogs with various β-amino-acid residue carbon chain lengths were evaluated using the cell-based assay against a TGA-type PTC (Table 3). Interestingly, the derivative **14** (TCP-112, Table 3), with a chain length one carbon shorter than that of **12**, exhibits stronger readthrough activity than **12**, with a ratio of 4.28 but no antimicrobial activity (MIC $\geqslant$ 1024 μg ml^{-1}) against *Staphylococcus aureus* NBRC13276 or *Escherichia coli* NBRC3972. Furthermore, the dose-dependent behavior of **14** was confirmed in the same cell-based assay (Figure 5). Next, the effect of the β-amino group was explored by synthesizing a series of α-amino derivatives with different chain lengths. The readthrough activities of these α-amino derivatives **34a–d** were found to be noticeably decreased (0.99–1.30). These results suggest that the amino group in position 3 on the alkyl side chain is important for activity. These modifications led to the discovery of a new derivative **14** with a more potent readthrough activity than **10** and **12**.

Even more potent readthrough derivatives were sought by performing a SAR study focused on the C terminus of **14**, introducing modifications that increased the hydrophobicity of the resulting

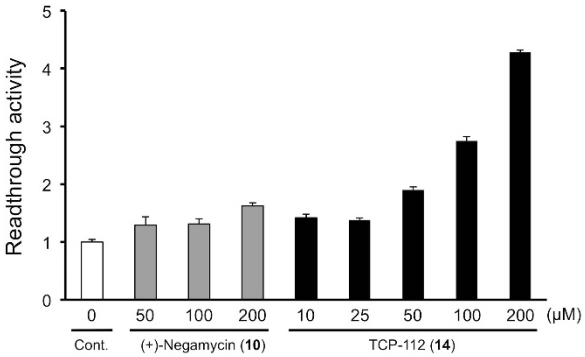
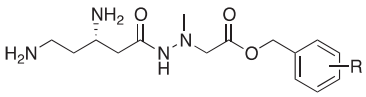


Figure 5 The dose-dependent manner of cell-based readthrough activity of TCP-112 (**14**) against TGA-type PTC. Cell, COS-7. Error bars indicate ± s.d. ($n=3$).

Table 4 Cell-based readthrough activities of the ester derivatives 15 and 39–43



Compound	R	ClogP[a]	Readthrough activity[b]
39	*o*-Br	0.78	3.83 ± 0.16
40	*m*-Br	0.78	3.75 ± 0.31
41	*p*-Br	0.78	2.98 ± 0.40
42	*o*-Cl	0.63	3.34 ± 0.15
TCP-182 (**15**)	*m*-Cl	0.63	4.90 ± 0.21
43	*p*-Cl	0.63	4.21 ± 0.16

Compounds were evaluated at a concentration of 200 μM. Data represent the mean ± s.d. ($n=3$).
[a]Values of the calculated log *P* (Clog*P*) determined using CS ChemBioDraw Ultra 12.0.
[b]Readthrough activity relative to control (=1) in COS-7 cells.

derivatives. The hydrophilicity of **10** and its derivatives tends to decrease cell penetration. We tested certain derivatives in which the carboxylic acid was replaced for an ester or an amide, reducing the hydrophilicity. The readthrough activity of the ethyl ester **35** was approximately half of that of **14**. The benzyl ester derivative (**36**) maintains a readthrough activity comparable to that of **14**. The amides **37** and **38**, which bear similar alkyl groups, failed to show any readthrough activity. The differences between the activities of the ester and amide suggested that the ester derivative acted as a prodrug to produce the corresponding carboxylic acid (**14**) during the cellular assay.

As the benzyl ester (**36**) shows potent activity, we prepared derivatives in which functional groups were introduced as substituents on the phenyl ring. The calculated log *P* (Clog*P*) value for each derivative was calculated using CS ChemBioDraw Ultra 12.0 as a hydrophobicity measurement. As shown in Table 4, the introduction of a bromine atom to the phenyl ring in **39**, **40** and **41** slightly decreased the activities compared with the benzyl ester derivative (**36**).

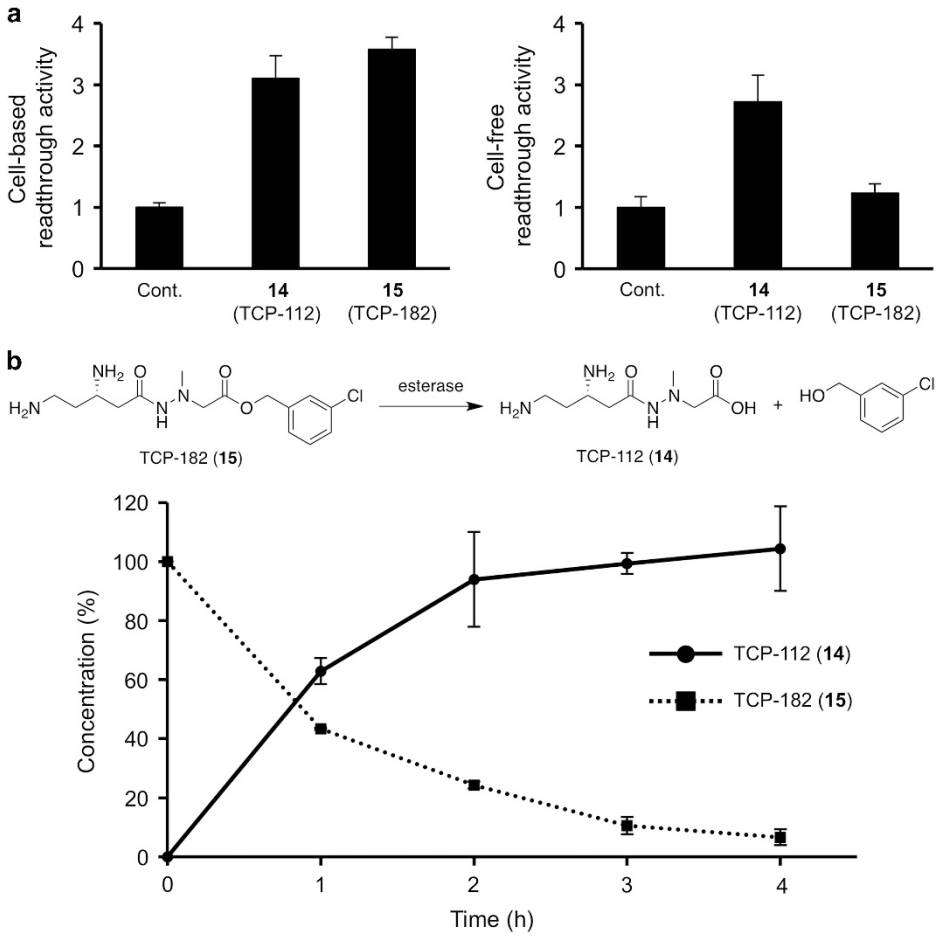


Figure 6 (**a**) Readthrough activities in cell-based and cell-free systems. Readthrough activity (ratio) relative to control (=1). COS-7 cell. Compound concentration, 200 μM (cell-based assay, $n=3$) or 20 μM (cell-free assay, $n=3$). Error bars indicate ±s.d. (**b**) Hydrolysis of TCP-182 (**15**) in the presence of porcine liver esterase. Compound concentration (%) was determined with RP-HPLC. Error bars indicate ±s.d. ($n=3$).

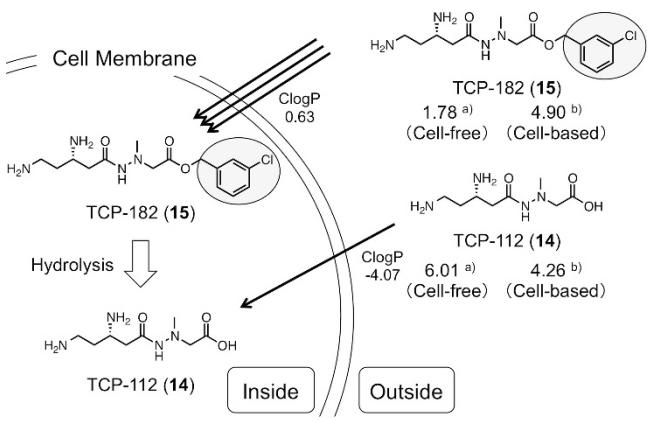


Figure 7 Tentative hydrolysis mechanism of conversion of prodrug TCP-182 (**15**) to the parent derivative TCP-112 (**14**). [a]Cell-free (human cell lysate) and [b]cell-based (COS-7 cell) readthrough activity compared with control (=1). Compound concentration, 20 μM (cell-free assay) or 200 μM (cell-based assay).

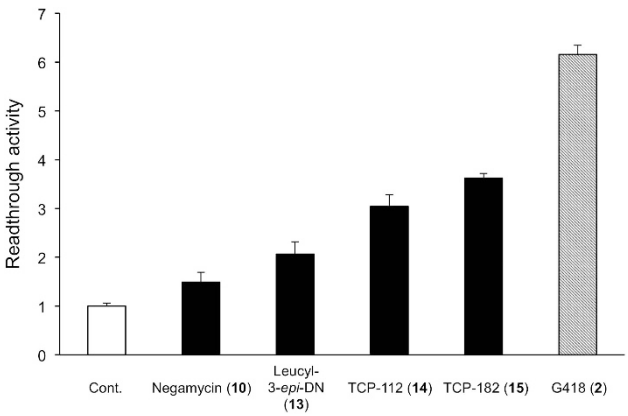


Figure 8 Cell-based readthrough activities (compared with control) of derivatives identified through the structure–activity relationship (SAR) studies of negamycin focused on the C5 position. Cell, COS-7. Compound concentration, 200 μM. Error bars indicate ±s.d. ($n=3$).

The *meta*-chloro-substituted derivative **15** (TCP-182), however, shows improved activity over **14**. The Clog*P* values of the bromine- and chlorine-substituted derivatives were calculated to be 0.78 and 0.63, respectively, indicating that the hydrophobic properties of these compounds were improved over that of **14** and **36** (Clog*P*; −4.07 and 0.08, respectively).

Because **14** exhibits both potency and hydrophobicity, we examined whether it could serve as a prodrug using a cell-free readthrough assay

(TGA) system[62] that directly evaluates the activity of compound **14** without the influence of cell membrane permeability. As shown in Figure 6a, the cell-free readthrough activity of the *m*-Cl benzyl ester (**15**) decreased markedly (1.23) compared with that obtained from the cell-based assay (3.58). As TCP-112 expresses potent readthrough activity in both cell-based and cell-free systems (3.10, 2.72, respectively), these results clearly suggest that **15** acts as a prodrug that was hydrolyzed to **14** intra- or extracellularly in the cell-based assay. The slightly higher activity of **15** compared with **14** in the cell-based assay may be due to the slight increase in the cell penetration ability. These results suggested that a prodrug strategy involving the carboxylic acid part may contribute to the readthrough activity.

To further understand whether **15** acts as a prodrug, the metabolites of **15** treated with porcine liver esterase in 100 mM phosphate buffer (pH 7.4) at 37 °C were studied. After incubation, we confirmed the time-dependent production of **14** along with the degradation of **15** (Figure 6b). These results suggest that the substituted benzyl ester derivatives function as a prodrug to produce the parent drug **14** in a living cell system (Figure 7). Finally, as shown in Figure 8, we summarized the readthrough activity of derivatives identified through the SAR studies focused on C3 and C5 positions.

CONCLUSIONS

We considered the readthrough activity of (+)-negamycin analogs in connection with the development of chemotherapies for nonsense-mediated genetic diseases. A medicinal chemistry approach to the discovery of novel potent candidates based on the negamycin structure was pursued by developing a total synthesis of (+)-negamycin (**10**), starting from N-Boc-glycinal (**16**). An overall yield of 42% was obtained over eight steps.[47] This route provides the most efficient strategy for preparation of **10**. Another synthetic strategy starting from a commercially available chiral ester and relying on a key intermediate was found to provide **10** in 13 steps with an overall yield of 31%.[52] An SAR study focused on the 3-amino group of **10** was conducted in transgenic mice to measure the mild *in vivo* readthrough activity of the 3-deamino derivative (**11**). Biochemical and immunohistochemical studies demonstrated that **11** restores some dystrophin expression in *mdx* mice and decreases serum creatine kinase levels, suggesting that the drug protects muscular tissue from collapsing. Compound **11** was also shown to have a lower toxicity profile than **10**, an essential feature for any long-term treatment of DMD.[55] The synthetic natural analogs 3-*epi*-deoxynegamycin (**12**) and its leucine adduct (**13**) display potent readthrough activities against nonsense mutations in eukaryotes but not in prokaryotes, and these compounds displayed no antimicrobial activity.[62] Most natural products identified previously with readthrough activity were discovered using primary antibiotic-screening assays that were not designed to select for readthrough activity alone. Therefore, the discovery of **12** and **13** as a readthrough compound suggests that many natural product readthrough compounds, so far unidentified and potentially without antimicrobial activity, may be discovered with a primary readthrough-screening assay. A SAR study based on the structure of **12** led us to identify the more potent derivative (**14**; TCP-112) and its ester prodrugs. **15** (TCP-182) appears to function as a prodrug in the living cell system and in *in vitro* studies.[63] These new derivatives constitute promising therapeutic candidates for the treatment of nonsense mutation-mediated genetic diseases such as DMD. Additional SAR studies are underway in an effort to develop more efficient synthetic analogs. The chemical biology underlying the readthrough mechanism is also being investigated.

DEDICATION

This paper is dedicated to Professor KC Nicolaou for his great scientific contribution to total synthesis of highly complex and biologically important natural products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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